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# Engineered Mesenchymal Stem Cells as Therapeutic Vehicles for the Treatment of Solid Tumors: Wnt and Hedgehog Biology-based Targeting and Transgene Expression

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Meiner Familie

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## Zusammenfassung

Diese Studie verbessert das Verständnis von Differenzierung und Rekrutierung mesenchymaler Stammzellen (MSC) im Kontext der Tumorstromabildung. Dieses ist eine wichtige Voraussetzung für die therapeutische Verwendung dieser Zellen, zum Beispiel in der Gentherapie. Um die therapeutische Anwendung von MSCs spezifischer gegen Tumorgewebe zu richten und dadurch Nebenwirkungen zu reduzieren, ist die bessere Erforschung der Rekrutierungs- und Migrationseigenschaften der MSCs notwendig. Es gibt Hinweise, dass wichtige Aspekte der MSC Biologie dem Wnt Signalweg unterliegen. In vitro wurde der Einfluss von Prekonditionierung humaner MSCs auf ihr Migrationsverhalten gemessen. Nach 24h und 48h Prekonditionierung von primären MSCs mit rekombinanten humanen Wnt3a Protein, welches den kanonischen Wnt Signalweg aktiviert, wurden signifikant zielgerichtetere Migrationseigenschaften im Vergleich zur unstimulierten Kontrolle gemessen. Nach weiterer Kultivierung der prestimulierten MSCs für zwei bis drei Passagen, wurden keine erhöhten Parameter für gerichtete Migration mehr gemessen. Daraus lässt sich schließen, dass der chemotaktische Effekt der Wnt3a Prestimulierung auf MSCs temporär und reversibel ist.

Der Wnt und Hedgehog (HH) Signalweg sind hochkonservierte Signalkaskaden, die in Karcinogenese und Differenzierung eine wichtige Rolle spielen. Liganden dieser Signalwege werden autokrin, parakrin und juxtakrin im Tumormilieu sezerniert und können diverse Zellen im Tumor aktivieren. Deshalb wurden im zweiten Teil dieser Arbeit spezifische synthetische Promotoren für die genannten Signalwege, auf ihre mögliche Aktivität im Tumormilieu untersucht – als Mechanismus für die potentiell erhöhte Expression von Zielgenen im Tumormilieu. Als erster Schritt wurde die jeweilige Promotoraktivität im experimentellen Tumormilieu charakterisiert. Mit Hilfe des Sleeping Beauty Transposon Systems wurden stabile Reporterzelllinien generiert, die *Gaussia* Luciferase als Korrelat für Promotoraktivität in den Überstand sezernieren. Hek293 Zellen wurden als einfach zu kultivierende und leicht zu transfizierende Zellen zur Prüfung der Konzepttauglichkeit verwendet. Hek293 Zellen die stabil mit Wnt oder HH Reportertransgenen transfiziert wurden, zeigten erhöhte Promotoraktivität in Kokultur mit Tumor- und Makrophagenzelllinien; auch

Tumorzell- und Makrophagenüberstände konnten die Hek293 Reporterzellen aktivieren. Im Gegensatz dazu, konnte in immortalisierten mesenchymalen Stammzellen (iMSC), die mit den gleichen Transgenen stabil transfiziert wurden, kein erhöhtes Signal gemessen werden – weder in Kokultur, noch mit konditionierten Überständen. Exposition der iMSC Reporterzellen mit aufkonzentriertem Makrophagenüberstand, zeigte signifikant erniedrigte Reporteraktivität. Zusammenfassend kann man sagen, dass Wnt- und HH-Reportertransgene im Tumormilieu beeinflusst werden, deren Auf- oder Abregulierung jedoch von der Reporterzellline abhängig ist. Aus den vorliegenenden Daten lässt sich schließen, dass mehr Signalwege getestet werden müssen, um einen geeigneten Promotor für die spezifische Expression von (Therapie-)genen in stabil transfizierten MSCs im Tumormilieu zu identifizieren.

Um das Tumormilieu und dessen möglichen Einfluss auf MSC zu erforschen, wurde es zum Ziel gesetzt Reporterzellen mit einem fluoreszierenden Protein als Korrelation zur Promotoraktivierung zu etablieren. Diese könnten dazu verwendet werden in einem 3D Tumormodel, einem sogenannten Tumorspheroid, nicht nur die Invasionsfähigkeit, sondern auch die Beeinflussung der Promotoraktivität währenddessen, in den verschiedenen Zonen des Tumormodels, zu messen. Stabil transfizierte iMSC und primäre MSC mit einem Wnt-Reportertransgen, dessen Expression an das fluoreszierendes Protein Tomato gekoppelt war, wurden etabliert. Die Funktionalität der Wnt-Reporterzellen wurde durch erhöhte Fluoreszenz nach Wnt3a-Stimulation in der FACS Analyse gezeigt. Reporter-iMSC wanderten in ein Tumorspheroid ein und die Fluoreszenz konnte mit einem Lichtscheibenmikroskop gemessen werden. Es lagen aus anderen Experimenten Hinweise vor, dass in iMSC und MSC diese Wnt-Reporterelemente durch Tumormilieu nicht stimuliert werden konnten. Daher war die Etablierung dieses Assays mit Wnt-Reporter-Plasmiden nicht erfolgreich. Da das fluoreszierende Protein Tomato über viele Passagen hinweg exprimiert wurde und Aufgrund seiner Leuchtkraft gut in unserem Fluoreszenzmikroskop und FACS detektiert werden konnte, bietet sich ein neuer Etablierungsversuch mit diesem Protein und einem anderen Reportertransgen an.

## Summary

This study improves our understanding of the mechanisms of mesenchymal stem cell (MSC) differentiation and recruitment in the context of tumor stroma formation, an important prerequisite for the therapeutic utilization of MSCs, for example as gene delivery vehicles. In order to reduce side effects, and to make gene delivery more targeted to tumors, the behavior of MSCs in response to tumor-derived signals was observed under different conditions. Wnt signaling is thought to underlie important aspects of MSC biology. The effect of prestimulation of human MSCs with the canonical Wnt activator protein Wnt3a on migration towards tumor conditioned medium was studied. After 24h and 48h of prestimulation of primary MSCs with recombinant human Wnt3a, various parameters indicated enhanced directed migration. No memory effect was observed, suggesting that the prestimulated cells did not show enhanced chemotactic parameters after culture for two to three passages. Therefore, Wnt3a prestimulation exhibits temporary effects on MSCs which are fully reversible.

Canonical Wnt and Hedgehog (HH) signaling are highly conserved pathways involved in carcinogenesis and differentiation. The ligands triggering these pathways are secreted into tumor environments, and through autocrine, paracrine or juxacrine signaling, can activate the diverse cells within tumors. In the second part of this thesis, the potential activation of synthetic Wnt and HH promoters in primary MSCs were evaluated as a mechanism for the potential induced expression of target genes in the tumor milieu. As a first step to characterize the potential promoter activity in response to experimental tumor-derived signals, a set of Sleeping Beauty transposon-based reporter plasmids were generated for stable integration of complex transgenes into target cells that use *Gaussia* Luciferase secretion into the growth media as a read-out for promoter activity. Because Hek293 cells are easy to cultivate and transfect, this cell line was used in control experiments. In in vitro experiments Hek293 cells were stably transfected with Wnt- or HH-responsive transgenes. The resulting lines showed elevated signaling activity in co-culture and with supernatant of various tumor cell lines and a macrophage cell line. In contrast, an immortalized MSC cell line (iMSC) stably transfected with the respective reporter plasmid did not show induction, rather a decreased signal.

Exposure of reporter iMSCs to concentrated supernatant from a macrophage cell line showed significantly decreased reporter activity. In conclusion, Wnt- and Hedgehog-responsive reporters engineered into cell lines can be positively and negatively influenced by tumor-relevant signals depending on the reporter line. Therefore, more pathways need to be tested to find the most suitable promoter for (therapeutic) gene expression in MSCs in the tumor milieu.

In order to investigate the tumor milieu and its potential effect on MSCs, reporter cells with a fluorescent protein read-out were established to measure promoter activity of stably transfected MSCs while invading a 3D tumor model, a so-called tumor spheroid. Changes in reporter activity would indicate pathway specific influences on MSCs exposed to various chemokine and oxygen gradients in the tumor spheroid. Primary MSC and iMSC with a Wnt-responsive element linked to fluorescent protein expression were established; Fluorescence activated cell scanning (FACS) analysis showed increased fluorescence after Wnt3a stimulation. Reporter iMSC invaded a tumor spheroid and fluorescence could be detected in a light sheet microscope. No evidence of reporter transgene activation in primary MSCs or iMSCs in the tumor milieu made the establishment of this assay with those pathway specific elements unsuccessful. However, the fluorescent protein Tomato was stably expressed over many passages and could be easily detected in our fluorescence microscope and FACS because of its brightness. When trying to establish this assay with another pathway specific element – known to be activated in MSCs in the tumor milieu – Tomato would be a suitable fluorescent protein.

# Introduction

## Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are pluripotent cells found in the bone marrow and other tissues including; peripheral blood, skeletal muscle, umbilical cord, amniotic fluid, liver, adipose tissue and dental pulp (Ullah, Subbarao, & Rho, 2015). No single markers exist for the characterization of MSCs, the minimal criteria for defining multipotent mesenchymal stem cells as outlined by the International Society for Cellular Therapy is defined by their expression of CD105, CD73 and CD90, which are associated with stromal cell biology, and the lack of expression of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR surface molecules which are associated with hematopoietic or endothelial cells. MSCs are thus defined as non-hematopoietic, multipotent stem cells, which are able to differentiate into adipose, bone and cartilage tissue and to grow adherently on plastic surfaces (Dominici et al., 2006). MSCs can show extensive heterogeneity.

Research on MSCs is a relatively new field; MSCs were first described in 1974 by Friedenstein et al. as a population of cells derived from bone marrow that had the appearance of fibroblasts and that could differentiate into adipocytes, osteocytes and chondrocytes (Friedenstein et al., 1974). In the 1990s these cells were termed mesenchymal stem cells.

### MSC homing to damaged tissue

In their role as mediators of tissue repair, MSC become mobilized from various niches and undergo directed migration to damaged tissue (J. F. Ji, He, Dheen, & Tay, 2004; Satake, Lou, & Lenke, 2004). Because solid tumors resemble chronically damaged tissue with extensive hypoxia, necrosis and inflammation, MSC are actively recruited to growing tumors where they can act as progenitors for cells that make up the tumor stroma (Studeny et al., 2002). Adoptively applied MSC have been shown to undergo recruitment of a variety of experimental tumors including; gliomas, melanoma, breast cancer, colon cancer, hepatocellular carinoma and liver metastasis of colon cancer, as reviewed by Bayo et al. (Bayo et al., 2014).

Despite the considerable amount of literature describing the phenomenon of MSC migration, the underlying mechanisms of MSC recruitment are not fully understood.

It is generally assumed that MSC homing follows the same steps described for leukocyte homing, e.g. mediated through integrins, selectins, chemokines and adhesion molecules (Figure 1).

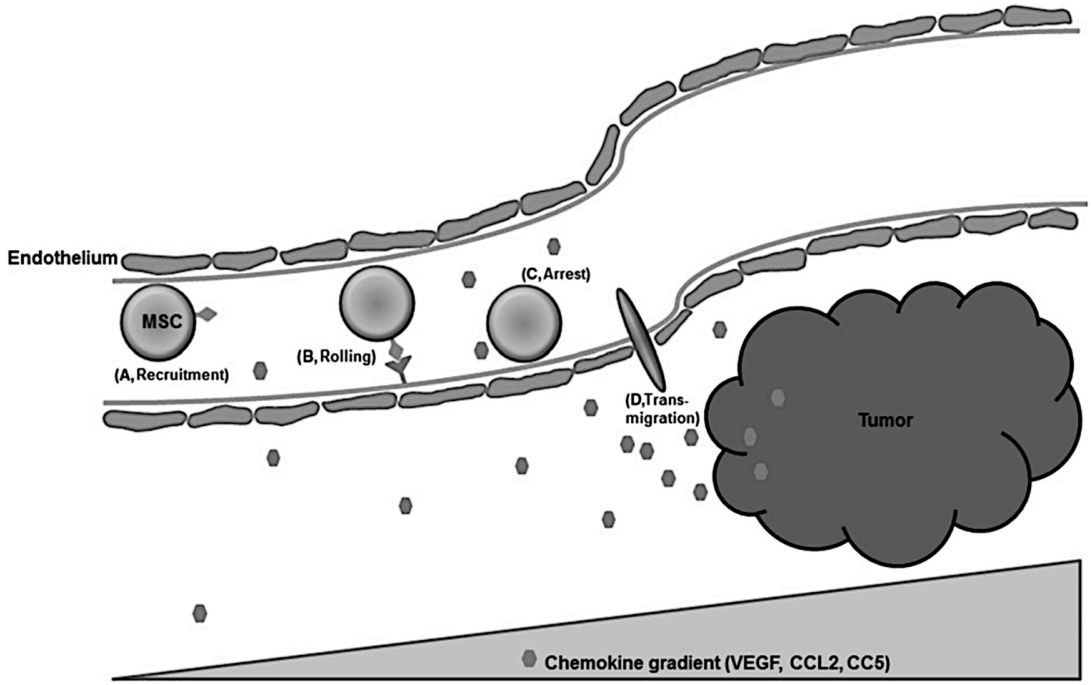



**Figure 1. Mesenchymal stem cell (MSC) recruitment to tumor milieu**

*MSCs are recruited to the tumor milieu by various factors, including chemokine gradients. Although the homing mechanism is not yet fully understood, it has been suggested that certain cytokines and chemokines (VEGF, CCL2, and CCL5/RANTES), as well as ANGPT2, released into the peripheral circulation help guide cells to tumors. A) recruitment, B) rolling, C) arrest within the vasculature, and D) transmigration across endothelium may be mediated by the mechanisms and adhesion molecules also direct leukocyte homing and adhesion cascade. Abbreviations: ANGPT2, angiopoietin 2; CCL, C-C chemokine ligand; MSC, mesenchymal stem cell; VEGF, vascular endothelial growth factor (Keung, Nelson, & Conrad, 2013).*

Several important cytokine/receptor pairs for MSC homing and transmigration have been identified, these include: SDF-1/CXCR4, SCF-c-Kit, HGF/c-Met,

VEGF/VEGFR, PDGF/PDGFr, MCP-1/CCR2, and HMGB1/RAGE as reviewed by Momin et al (Momin, Mohyeldin, Zaidi, Vela, & Quinones-Hinojosa, 2010). MSCs express CXCR4 and c-met receptors, and can be attracted by stromal-derived factor (SDF-1) and hepatocyte growth factor (HGF) gradients in vitro. Moreover, the expression of matrix metallo- proteinase-2 (MMP-2) is downregulated in MSCs when they contact tumor conditioned medium. SDF-1 and HGF are upregulated in damaged tissue (Son et al., 2006).

CXCR4 expression can be detected in MSCs, but often not on the surface (Von Luttichau et al., 2005), whereas Lourenco et al. suggest that tumor conditioned-medium can trigger the expression of intracellular stored CXCR4 on the cell surface, thus improving the response to a chemotactic gradient, independently of gene expression (Lourenco et al., 2015). Macrophage migration inhibitory factor (MIF) may also be a key player in MSC migration towards tumor conditioned-medium, acting through the CXCR4 receptor (Lourenco et al., 2015). Ponte et al. also concluded that an inflammatory milieu enhances MSC migration. In a transwell migration assay, MSCs prestimulated with tumor necrosis factor α (TNFα) showed enhanced migration compared to unstimulated control, however, interleukin-1β did not alter the migratory ability (Ponte et al., 2007). Moreover, TNFα prestimulated MSCs also showed increased migration capacity towards the chemokines RANTES/CCL5, SDF-1/CXCL12, Macrophage-derived chemokine (MDC) and the growth factor angiopoietin-1, as compared to unstimulated MSCs (Ponte et al., 2007).

Understanding MSC homing and finding ways of enhancing their tropism for solid tumors is important for enhancing the therapeutic application of MSCs. There are various approaches that have been suggested to modulate the homing efficiency of MSCs these include; the use of culture conditions or prestimulation to enhance the expression of homing molecules, engineering of novel cell surface receptors to improve homing, optimizing the mode of administration of MSCs to an organism and the modification of target tissue to better attract MSCs.

Various factors have been evaluated to precondition MSCs in vitro, also called MSC priming. Effects on the cells' behavior, including homing potential, and on

surface molecules, indicating differentiation, have been reported (Lu, Haider, Jiang, & Ashraf, 2009). Moreover, MSCs precultured under hypoxic conditions prior to transplantation were shown to improve their tissue regenerative potential in a mouse model, and enhanced their migration rate in vitro (Rosova, Dao, Capoccia, Link, & Nolta, 2008).

The signaling network controlling MSC homing is complex and not yet fully understood, it may show differences based on target tissues. In vitro models were used to observe the migratory behavior of MSCs. Aiming at better understanding and improving MSCs tumor homing not only in vitro, but also potentially in vivo, varying prestimulation conditions were evaluated.

The aforementioned studies evaluated several important signaling molecules for their influence on MSC migration. Wnt and Hedgehog pathways are associated with the migratory capacity of cells. Thus, the effects of modulation of these pathways could be monitored in part through quantification of migratory capacity. To date, the effect of canonical Wnt or Hedgehog pathway activation on the tropism of MSC for tumor signals has not been described.

**MSCs within the tumor milieu**

In addition to malignant cells, the tumor milieu is also composed of inflammatory cells (neutrophils, macrophages, mast cells and lymphocytes), newly formed blood vessels, and a large number of fibroblasts and myofibroblasts which together are referred to as the tumor stroma. The tumor stroma effects cancer progression, for example, by driving tumor cell proliferation, angiogenesis and metastasis.

The tumor environment has been described as resembling a wound that does not heal (Dvorak, 1986; Schafer & Werner, 2008). MSCs have been shown to act as precursor cells for many of the cells that make up the tumor-associated stromal component. Within the tumor microenvironment, MSCs have been shown to give rise to carcinoma associated fibroblasts (CAF), pericytes, endothelial cells, and fibrovascular stroma (Mishra et al., 2008).

Tumor-resident endothelial cells and stromal cells produce various chemokines and growth factors, such as CCL2, CCL5, and angiopoietin 2 (Keung et al., 2013). MSCs

can respond to angiogenesis signals by secreting various factors such as leukemia inhibitory factor, macrophage colony-stimulating factor, macrophage inflammatory protein-2 and vascular endothelial growth factor (VEGF) (Suzuki et al., 2011). MSCs are seen as key players in generating and moderating the tumor microenvironment, thus, genetically modified MSCs constitute a potential therapeutic vehicle.

MSCs are thought to be tumor promoting as well as immunoregulatory. They can be immune suppressive, for this reason it has been suggested that they could help the tumor escaping from immune surveillance (Han et al., 2012). MSCs have been described to suppress a variety of immune cells, including dendritic cells, natural killer cells, and B- and T- lymphcytes (Glennie, Soeiro, Dyson, Lam, & Dazzi, 2005; Jiang et al., 2005). In the tumor microenvironment, MSCs promote tumor cell development and progression. MSCs in coculture with ovarian cancer cells, were shown to lead to altered biological function of gene clusters known for increased metastatic ability, proliferation and chemoresistance (Lis et al., 2012).

## Therapeutic strategies involving MSCs

In contrast to embryonic stem cells, MSCs are free of ethical concerns and can be harvested from numerous tissue sources. These cells also show low immunogenicity and low teratoma risk. MSCs can differentiate into various cell lineages, they secrete soluble factors crucial for cell survival and proliferation, they can modulate immune responses, and migrate to sites of tissue injury (Figure 2). These characteristics have led to research directed toward the potential clinical application of MSCs.

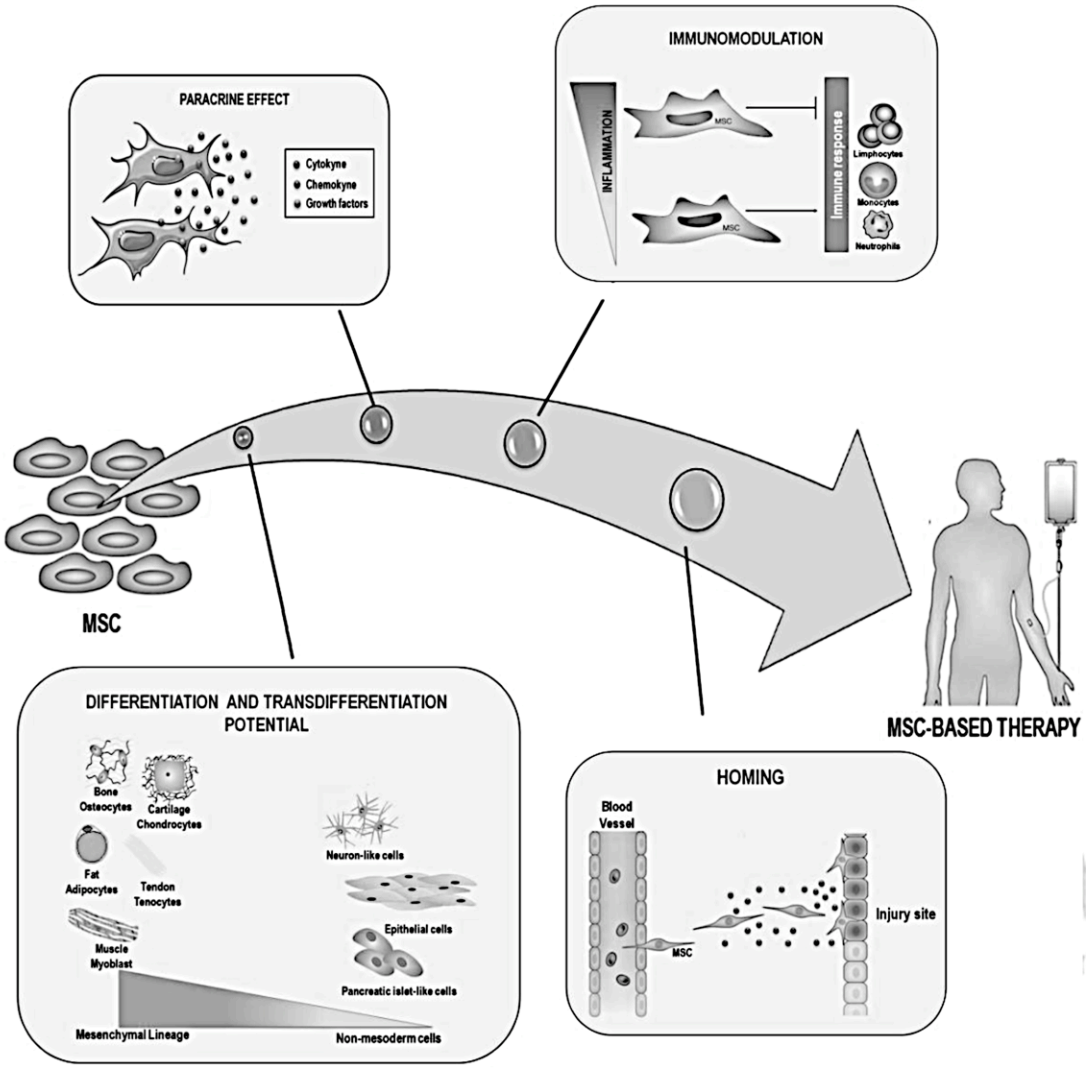



**Figure 2 Mesenchymal stem cell (MSC) properties qualifying them for therapeutic applications**

*The properties of MSCs that make them useful as therapeutic vehicles. In addition to being able to differentiate into various tissues, MSCs exert a paracrine effect, are immunomodulatory, and show homing to tumor and sites of injury (Squillaro, Peluso, & Galderisi, 2015).*

In June 2015, 493 MSC-based clinical trials, either completed or ongoing, were available in the database of the US National Institute of Health. These trials include the application of these cells to treat hematological disease, graft-versus-host

disease, organ transplantation, diabetes, inflammatory diseases, and diseases in the liver, kidney, and lung, as well as cardiovascular, bone and cartilage, neurological, cancer and autoimmune diseases (Squillaro et al., 2015).

Human MSCs do not induce alloreactivity in vitro or in vivo and hematopoietic stem cell transplantation recipients show reduced donor-specific immune responses after treatment with third-party MSCs (Sundin et al., 2009).
The ability of MSCs to traffic from bone marrow or peripheral blood into damaged tissues to help effect repair makes them suitable for diverse clinical applications. Because these cells are involved in wound healing (Mackenzie & Flake, 2001), they support tissue regeneration after myocardial infarction (Kawada et al., 2004) and they promote restoration of bone marrow after myeloablative chemotherapy (Koc et al., 2000). Importantly, MSCs also home to tumor microenvironments that are seen as chronic wounds by the body (Nakamizo et al., 2005).

Their general tropism for tumors and their efficient integration at the tumor site make MSCs an attractive therapeutic vehicle for the delivery of nanoparticles, oncolytic viruses or therapeutic genes to solid tumors.

**Tumor therapy with genetically modified MSCs**

MSCs have been used as vehicles to shuttle diverse therapeutic genes into critical parts of growing tumors. This has been referred to as a ‘Trojan horse’-based therapy approach (Bao et al., 2012). There are various strategies that have been used to genetically modify MSCs to target tumor biology (Table 1).

**Table 1 Examples of genetically engineered MSCs targeting tumor biology**

| Therapeutic gene system | Examples | Literature |
|---|---|---|
| **Proteins**<br>**- cytotoxic**<br>**- antiangiogenic** | -Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)<br>-human pigment epithelium-derived factor (PEDF)<br>-NK4 (HGF antagonist) | (Loebinger, Eddaoudi, Davies, & Janes, 2009)<br>(Gao et al., 2010)<br>(Kanehira et al., 2007) |
| **Immunostimulation** | -Interferon α<br>-Interferon β<br>-Interleukin-2<br>-Interleukin-12 | (Ren et al., 2008)<br>(Nakamizo et al., 2005)<br>(Stagg, Lejeune, Paquin, & Galipeau, 2004)<br>(Eliopoulos, Francois, Boivin, Martineau, & Galipeau, 2008) |
| **Gene-directed enzyme-producing therapy (GDEPT) utilizing suicide genes** | -Thymidine kinase / Ganciclovir (GCV)<br>-Cytosine deaminase / 5-fluorocytosine<br>-Cytochrome P450 isoforms / cyclophosphamide or ifosfamide<br>-Tomato thymidine kinase 1 / zidovudine | (Zischek et al., 2009)<br>(Kucerova, Altanerova, Matuskova, Tyciakova, & Altaner, 2007)<br>(Chen et al., 2004)<br>(Stedt et al., 2015) |
| **Sodium iodide symporter (NIS) gene** | $^{131}I$, $^{123}I$, $^{125}I$, $^{124}I$, $^{99m}Tc$, $^{188}Re$, $^{211}At$ | (Dwyer et al., 2011; Knoop et al., 2011) |

Gene-directed enzyme-producing therapy (GDEPT) systems have been used to engineer MSC to express ‘suicide genes’. These enzymes metabolize prodrugs into tumor toxic agents. High regional doses of a therapeutic drug are produced based on expression of the suicide gene. The cytotoxic drug effects not only the cells where the drug is produced, but also neighboring cells that do not express the enzyme. This is called ‘bystander effect’ (Altaner, 2008; Matuskova et al., 2010). A number of enzyme–prodrug systems have been developed, including the herpes simplex thymidine kinase (HSV-tk)/Ganciclovir (GCV), cytosine deaminase/5-fluorocytosine, cytochrome P450 isoforms/cyclophosphamide or ifosfamide, and tomato thymidine kinase 1/zidovudine systems (Table 1).

The choice of enzyme–prodrug system should be based on the characteristics and biology of the tumor of interest. The more directed the suicide gene expression is to the tumor milieu, the less potential damage to healthy tissue. In order to make the antitumor effect more selective, MSCs can be engineered to express therapeutic transgenes only when they are activated within tumor environment. This selective transgene expression can be achieved by the use of tissue specific gene promoters/enhancers that are induced only when the MSC respond to signals within the tumor setting (Bao et al., 2012). Several different gene promoters have been tested in this capacity (Conrad et al., 2011; Guo et al., 2007; Niess et al., 2011; Nyati et al., 2002; Yan et al., 2014). In an early example of this approach, the carcinoembryonic antigen (CEA) promoter was used to express suicide genes in CEA positive colon cancer cell lines in combination with different enhancer elements (Guo et al., 2007; Nyati et al., 2002). The promoter region of α-fetoprotein was also used as a regulator for tumor-specific soluble TRAIL expression by MSC in an animal model of HCC that showed significantly inhibited tumor growth (Yan et al., 2014). Control of suicide gene expression has been demonstrated using the Tie2 promoter/enhancer. Tie2 is a cell surface receptor for the proangiogenic factor angiopoietin-1. Tie2-HSV-tk MSCs were shown to successfully treat experimental tumors in murine models of pancreatic, breast and liver cancer (Conrad et al., 2011; Niess et al., 2011). A second approach has made use of the promoter for the human RANTES/CCL5 gene which has showed promising results in diverse animal models (Niess et al., 2015; Zischek et al., 2009).

The first clinical trials to evaluate genetically modified MSCs for tumor therapy are currently underway. These include HSV-tk expressed under the control of the CCL5/RANTES promoter in autologous MSCs, where therapy is initiated by addition of Ganciclovir (Niess et al., 2015). Phase I study protocols have also been published for MSCs genetically engineered to express Interferon β (ClinicalTrials.gov number NCT02530047) as well as using MSCs for the delivery of an oncolytic virus (ClinicalTrials.gov number NCT02068794), both studies targeting ovarian cancer.

While these preclinical and clinical studies suggest a sound basis for this therapy approach, there are still obstacles that need to be overcome as we progress to the next stage of clinical development. After systemic intravenous application, many MSCs become entrapped in the microvasculature, especially of the lung. For intra-arterial injection microthrombi formation are also a concern. Therefore, optimizing the mode of MSC application is still an important issue.
Further research is also needed to better understand interactions between MSCs and the inflammatory, or tumor milieu. Localized therapeutic gene expression, in addition to enhancing tumor targeting, would also help to overcome potential side effects of MSC therapies. As canonical Wnt and Hedgehog signaling pathways are strongly linked to development and carcinogenesis, in this thesis gene promoters based on these pathways were evaluated for their selective activation by tumor-derived signals.

## Wnt signaling pathway

Wnt signaling pathways are highly conserved across metazoans and have important roles in various biological processes, including embryonic development, tissue homeostasis and carcinogenesis (Logan & Nusse, 2004). In 1982 Nusse and Varmus identified the first member of the Wnt-ligand family in the mouse, called Int1 (Nusse & Varmus, 1982). In 1987 Wingless was identified in *drosophila* as being essential for wing development (Cabrera, Alonso, Johnston, Phillips, & Lawrence, 1987). Rijsewijk et al. reported a Drosophila homolog of the mouse int-1 gene and showed that this gene is identical to Wingless (Rijsewijk et al., 1987). In the 1990s the nomenclature was changed to Wnt (for Wingless-related integration site) to denote genes belonging to the int1/Wingless family, and the founding member int1 was renamed as Wnt1 (Nusse & Varmus, 2012). Currently, there are three different intracellular Wnt signaling pathways described, the canonical/β-catenin dependent, the non-canonical/planar cell polarity (PCP), and the $Ca^{2+}$ dependent pathway. All three initiate signaling cascades via a ligand binding to a Wnt receptor belonging to the frizzled (Fz) receptor family. The Wnt family is comprised of 19 secreted glycoproteins with crucial roles in the regulation of diverse processes, including cell proliferation, survival, migration and polarity, specification of cell fate, and self-renewal in stem cells (Miller, 2002).

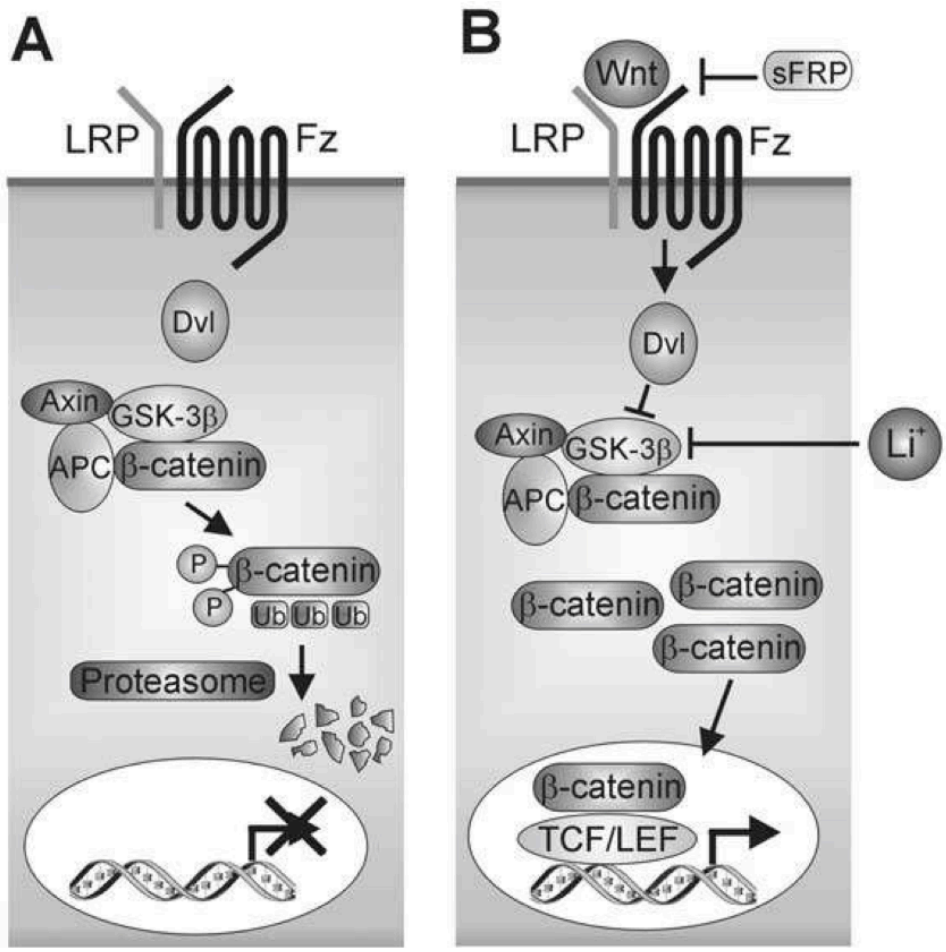



**Figure 3 The canonical Wnt signaling pathway**

*The protein β-catenin is a key player in canonical Wnt signaling activation. Generally it is identified in three distinct loci: at cellular adherent junctions, where it directly interacts with E-cadherin; in the cytosolic space; and in the nucleus. A): Unstimulated canonical Wnt signaling pathway. In most normal unstimulated adult cells, the Wnt/β-catenin pathway is inactive, and this is ensured by the absence of Wnt protein and the degradation of β-catenin. In the absence of Wnt ligand, β-catenin is associated with the so-called destruction complex formed by glycogen synthase kinase–3β (GSK-3β), axin, and adenomatous polyposis coli (APC). Following phosphorylation of β-catenin, it is marked for ubiquitinylation and subsequently degraded by proteasomes. B): Stimulated canonical Wnt signaling. Wnt ligand binding to Frizzled (Fz) receptor and lipoprotein receptor–related protein (LRP) coreceptor activates Disheveled (Dvl), which inhibits GSK-3β. β-catenin accumulates and associates with T-cell factor and lymphoid enhancer factors*

*(TCF/LEF) in the nucleus to regulate target gene transcription. Secreted Frizzled-related peptides (sFRPs) compete with Fz for Wnt ligand binding, thereby regulating signaling levels. Lithium (Li) inhibits GSK-3β, leads to downstrem Wnt signaling activation and therefore can be used to mimic canonical Wnt signaling (Etheridge, Spencer, Heath, & Genever, 2004).*

The canonical Wnt signaling cascade is shown in figure 3. Canonical Wnt signaling can be activated through various ligands, such as Wnt1, Wnt3, and Wnt3a that bind to one or more of the Fz receptors. Canonical Wnt signaling can be mimicked by the glycogen synthase kinase–3β (GSK-3β) inhibitor lithium chloride (LiCl) as it leads to downstream Wnt signaling activation. More recently several small molecules which modulate the signaling cascade have been identified (Kahn, 2014). Additionally, it has been suggested that in some cell types, stimulation with Wnt3a induces differentiation, for example during myogenesis and chondrogenesis (Bergwitz, Wendlandt, Kispert, & Brabant, 2001; Ridgeway, Petropoulos, Wilton, & Skerjanc, 2000).

The TCF/Lef1 transcription factors are the downstream targets of canonical Wnt signaling. These factors traffic to the nucleus and interact with β-catenin to moderate target gene expression. Because TCF/Lef1 recognizes a well-defined DNA binding site, it is possible to engineer luciferase-based Wnt reporters by multimerizing a binding site sequence (Korinek et al., 1997).

**Wnt signaling in MSC**

In 2004, canonical Wnt signaling was first described in human MSCs. An expression profile of Wnt signaling molecules in MSCs was initially identified using real-time PCR. MSCs express various Wnt ligands and Frizzled (Fz) receptors which suggest potential autocrine regulation of the pathway (Etheridge et al., 2004). Etheridge et al. detected expression of Fz receptors 2-6 in primary MSCs. Kolben et al. concluded that Fz 9 and Fz 10 are not expressed in MSCs while Fz 3 was expressed at low levels; the other Fzs exhibited high expression rates (Kolben et al.,

2012). However, one has to consider that the mRNA expression profile does prove functional receptors on the surface (Table 2).

**Table 2 Overview of real-time PCR analysis to identify expression of Wnt signaling receptors, Frizzled (Fz), in primary human MSCs**

| | (Etheridge et al., 2004) | (Kolben et al., 2012) |
|---|---|---|
| **Fz1** | - | ✔ |
| **Fz2** | ✔ | ✔ |
| **Fz3** | ✔ | ✔ |
| **Fz4** | ✔ | ✔ |
| **Fz5** | ✔ | ✔ |
| **Fz6** | ✔ | ✔ |
| **Fz7** | - | ✔ |
| **Fz8** | - | ✔ |
| **Fz9** | NA | - |
| **Fz10** | - | - |

Human primary MSCs tested for the presence of Wnt genes using real-time PCR showed expression of Wnt2, Wnt4, Wnt5a, Wnt11 and Wnt16. Wnt1, Wnt2b, Wnt3, Wnt3a, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt10a, Wnt10b, Wnt14 and Wnt14b were not detectable (Etheridge et al., 2004).

It has been proposed that low level stimulation using the canonical Wnt stimulators Wnt3a and LiCl, at a concentration of 1 and 4mM respectively, enhances the proliferation, and increases the ability of MSCs to transmigrate through transwell filters coated with human extracellular matrix. Moreover, neither osteogenic nor adipogenic differentiation of MSCs could be observed suggesting a differentiation of the MSC (Neth et al., 2006). Boland et al. also showed that in MSCs exposed to Wnt3a osteogenic differentiation is inhibited (Boland, Perkins, Hall, & Tuan, 2004).

## Hedgehog signaling pathway

Hedgehog (HH) signaling plays important roles in various biological processes, including embryonic development, tissue homeostasis, and regeneration. Through its regulation of epithelial-mesenchymal interactions, and subsequent control of organ system patterning and growth, HH signaling is critical for embryogenesis. Aberrant HH signaling is associated with cancer, notably basal cell carcinoma and medulloblastoma.

The name Hedgehog derives from the short and “spiked” phenotype of the cuticle of the HH mutant *drosophila* larvae. Mutations in the HH gene were first described in 1980 in *drosophila* (Nusslein-Volhard & Wieschaus, 1980), and in 1996 it was first associated with human cancer (Hahn et al., 1996). HH signaling is associated with many types of cancer – including skin, lung, brain, and gastrointestinal cancers as well as leukemia. There are canonical and non-canonical HH signaling cascades described.

### Canonical hedgehog signaling

Key components of HH signaling include the ligands Sonic hedgehog (Shh), Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Shh exhibits the widest tissue expression. Pachted1 (Ptch1) and Smoothened (Smo) are important regulators of HH signaling, negative and positive respectively; and the transcription factors downstream of the pathway are the glioma-associated oncogene (Gli) family members. Gli 1-3 - members of the zinc-finger containing protein family. Conserved HH targets include Gli1 and Ptch1, and their expression is commonly used to measure HH signaling activity. Gli1 functions as an activator, its transcription is induced by HH signaling and serves to amplify the HH signal. Hedgehog reporter plasmids have been generated through the multimerization of Gli protein binding sites (Kinzler & Vogelstein, 1990; Sasaki, Hui, Nakafuku, & Kondoh, 1997). Canonical HH signaling goes over PTCH-SMO-GLI axis (See figure 4). Despite intensive investigation, the exact mechanism is still unknown. Gli target genes include Cyclin-D1 (cell proliferation), ABCG2 (drug resistance), BCL2 (apoptosis resistance), Snail (EMT), WNT2 (Cell stem ness), VEGF (angiogenesis), TGFβ (immune modulation) (Jia, Wang, & Xie, 2015).

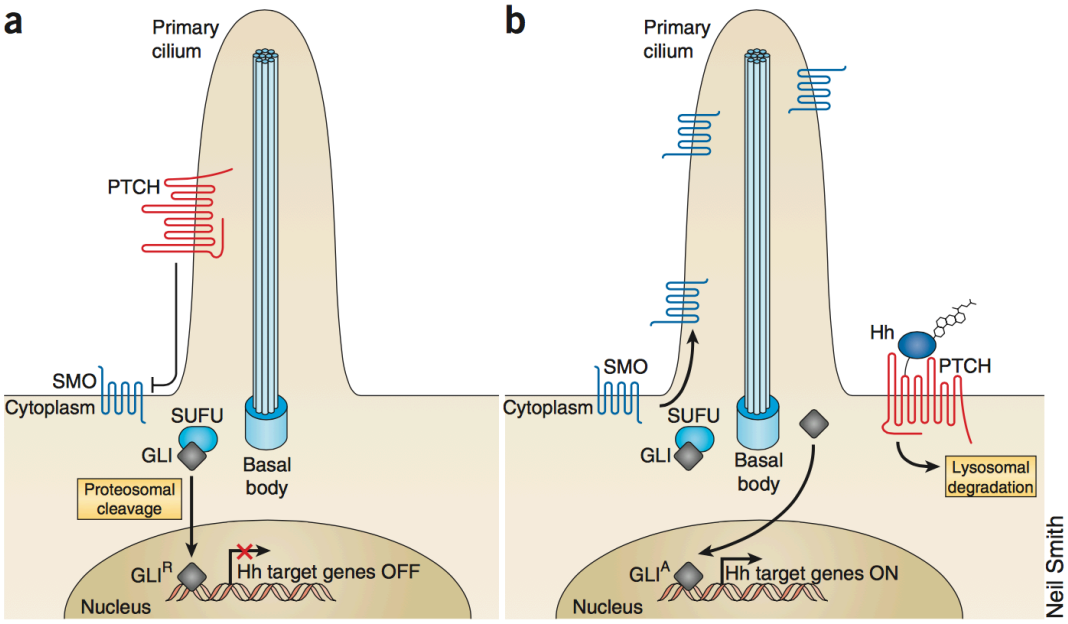



**Figure 4 Mammalian canonical Hedgehog (HH) signaling pathway**

*Transcription of HH target genes. a) In the absence of HH ligand, Pachted (Ptch) localizes in the cilia and represses Smoothened (Smo) activity by preventing its trafficking to the cilia. Glioma-associated oncogene (Gli) transcription factors are sequestered in the cytoplasm by several protein mediators, including protein kinase A (PKA), glycogen synthase kinase 3β (GSK-3β), casein kinase 1α (CK1-α) and suppressor of fused (SUFU). Gli undergoes proteasomal cleavage and the resulting repressor form (GLIR) translocates to the nucleus and inhibits translation of HH target genes. b) On ligand binding, Ptch is displaced from the cilia, thereby allowing ciliary accumulation and activation of Smo. Activated Smo orchestrates a signaling cascade that finally results in translocation of an activated form of Gli (GLIA) to the nucleus, where it induces expression of HH target genes (Amakye, Jagani, & Dorsch, 2013).*

### Noncanonical hedgehog signaling

By noncanonical, we collectively refer to cellular and tissue responses to the HH isoforms that are independent of transcriptional changes mediated by the Gli family of transcription factors (Robbins, Fei, & Riobo, 2012). However, the definition of noncanonical HH signaling is still heterogeneous. Because of an early agreement that all HH signaling involved Smo and Gli, noncanonical signal transduction is only

recently considered part of HH signaling. Noncanonical signaling routes have been associated with cancer, as they include genetic or expression changes of multiple oncogenes and tumor suppressors such as p53, KRAS, BRAF and PTEN.

**Hedgehog signaling pathway in MSC**

Relatively little is known about HH signaling in human MSC. This appears to be due to the differences in HH signaling in mammals and other animals, and to the relatively new interest in this pathway.

In real-time PCR experiments it has been shown that human MSCs express HH ligands (SHH and IHH), the HH receptor (PTCH), the HH co-receptor (SMO), and several HH-responsive target genes (GLI1-3) (Lin et al., 2008). By contrast, Kobune et al. could not detect SHH, although its expression was detected in other cell types. They suggested that the expression of IHH in human MSCs may be more significant than the expression of SHH (Kobune et al., 2004). It has been proposed that recombinant Shh induces proliferation and differentiation of MSCs (Warzecha et al., 2006).

## Rationale of this study

The central goals of this thesis were to investigate Wnt and Hedgehog signaling in the context of MSC biology, and in tumor milieu cross communication. To expand our understanding of these pathways in tumor stroma biology, and to provide insight into the future development of new molecular tools to refine the targeting of engineered MSC for tumor therapy (Figure 5).

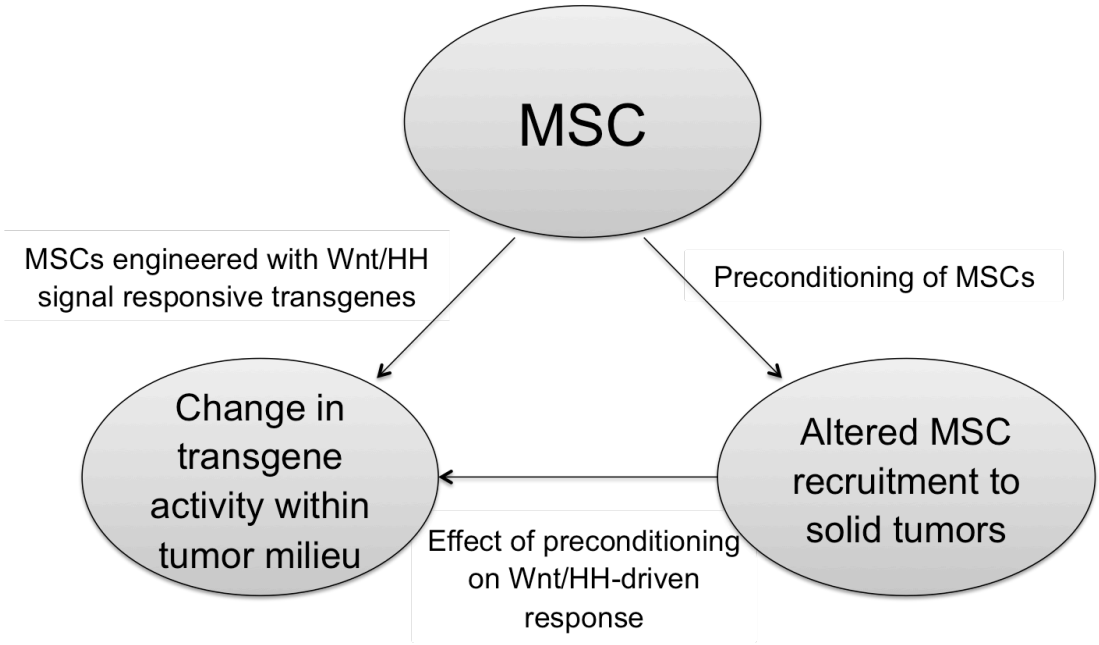



**Figure 5 Overview of this thesis' central research**

## Specific aims and scope

Canonical Wnt and Hedgehog signaling are important pathways in embryogenesis, cell differentiation, and tumor development. A better understanding of their activity in within the tumor milieu can be exploited for therapeutic applications. Focusing on improving cellular therapies based on engineered mesenchymal stem cells as therapeutic delivery vehicle, the following research questions were addressed:

- Does prestimulation of these pathways influence mesenchymal stem cells migratory potential towards tumor-derived signals?
- Is it possible to activate Wnt or Hedgehog pathway specific promoters in experimental tumor milieu?
- Is it possible to activate canonical Wnt and Hedgehog responsive elements stably transfected into mesenchymal stem cells based on the response of the MSC to signals within the tumor microenvironment?

# Materials

## General information

Permission for conducting gene experiments was obtained according to the 'Gesetz zur Regelung von Fragen der Gentechnik' of June 20, 1990 (Bundesgesetzblatt, 1990 I, page 1080).

Studies using human material comply with the ethics permission obtained by PD Dr. Irene von Lüttichau. MSCs were harvested from donated bone marrow samples.

## Cell culture

### Media and supplements

| | |
|---|---|
| **7-Aminoactinomycin D (7-AAD)** | Sigma-Aldrich, St.Louis |
| **Blasticidin** | InvivoGen, San Diego |
| **Bovine serum albumin (BSA)** | Invitrogen, Carlsbad |
| **CMFDA CellTracker™ Green** | Thermo Fisher, Waltham |
| **Collagen I bovine 5mg/ml** | Life Technologies, Carlsbad |
| **Doxycycline Hyclate** | Santa Cruz, Texas |
| **Dulbecco's Modified Eagle Medium (DMEM) (1x), liquid, Low glucose, contains sodium pyruvate** | Invitrogen, Carlsbad |
| **Dulbecco's Modified Eagle's Medium - low glucose 10x** | Sigma-Aldrich, St.Louis |
| **Dimethylsulfoxide (DMSO)** | Merck, Darmstadt |
| **Dulbecco´s PBS (without $Ca^{2+}$ and $Mg^{2+}$)** | PAA Laboratories, Pasching |
| **Fetal calf serum (FCS)** | Biochrom AG, Berlin |
| **L-Glutamin** | Thermo Fisher, Waltham |
| **Heparin-Natrium-25 000** | Ratiopharm, Ulm |
| **HEPES, 1 M** | Invitrogen, Carlsbad |

| | |
|---|---|
| **Hygromycin B** | Roche, Basel |
| **Insulin solution human** | Sigma-Aldrich, St.Louis |
| **$NaHCO_3$ (75g/l)** | Sigma-Aldrich, St.Louis |
| **NaOH** | Sigma-Aldrich, St.Louis |
| **Oxalacetic acid** | Fluka, Basel |
| **Penicillin/ Streptomycin (P/S), 100x** | PAA Laboratories, Pasching |
| **Pyruvic acid** | Sigma-Aldrich, St.Louis |
| **RPMI 1640** | Invitrogen, Carlsbad |
| **Trypan blue 0.4%** | Lonza, Basel |
| **Trypsin-EDTA in PBS, 1x** | PAA Laboratories, Pasching |

**Cell lines and primary cells**

| Cell line | Description | Source | Medium | Supplements |
|---|---|---|---|---|
| **Hek293** | Human embryonic kidney cells | M. Mack | DMEM | 10% FCS, 1% PS |
| **hbMSCAp172_1 (MSC)** | Primary mesenchymal stem cells derived from human bone marrow of a 37-year-old man | Isolation and expansion company Apceth GmbH & Co | DMEM | 10% FCS, 1% PS, 2IE/ml Heparin-Natrium and 5% platelets or medium provided by the company Apceth GmbH & Co |
| **HT1080** | Human fibrosarcoma cell line derived from a 35-year-old Caucasian man | Rechts der Isar, C. Kopiz | DMEM | 10% FCS, 1% PS |

| | | | | |
|---|---|---|---|---|
| **HT29** | Human colorectal adenocarcinoma (44-year-old caucasian woman) | A. Griffioen | DMEM | 10% FCS, 1% PS |
| **HUH7** | Human hepatocellular carcinoma cells (liver tumor from a 57-year-old man) | C. Spitzweg | DMEM | 10% FCS, 1% PS |
| **L87/4 (iMSC)** | SV-40 immortalized human mesenchymal stem cells derived from bone marrow (70-year-old male donor) | Pathology LMU | RPMI 1640 | 10% FCS, 1% PS |
| **MonoMac6 (MM6)** | Monocytic tumor cell line (isolated from a male patient with monocytic leukemia) | L. Ziegler-Heitbrock | RPMI 1640 | 10% FCS, 1% PS, MEM 1x, Insulin 900µg/ml, 1mM Oxalacetic acid, 5mM Pyruvic acid |
| **SW480** | Human colorectal adenocarcinoma (50-year-old male) | A. Griffioen | DMEM | 10% FCS, 1% PS |
| **V54/2 (iMSC)** | SV-40 immortalized | Pathology LMU | RPMI 1640 | 10% FCS, 1% PS |

| | |
|---|---|
| | human mesenchymal stem cells derived from peripheral blood |

In this thesis L87/4 and V54/2 were referred to as immortalized mesenchymal stem cells (iMSCs).
Experiments within the scope of this thesis with primary cells were conducted with human bone-marrow derived MSC in accordance with the ISCT criteria (Dominici et al., 2006). All primary MSCs used in this thesis were derived from the bone marrow of the same donor, the internal code name of these cells was hbMSCAp172_1.

## Bacteria

One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* from Invitrogen, Carlsbad

## Buffer and solutions

### Molecular biology

| | |
|---|---|
| **Buffers for DNA restriction enzymes, 10x** | NEB, Frankfurt |
| **Buffer TE, endotoxin-free** | Qiagen, Hilden |
| **Loading buffer for agarose gels, 6x** | 0.25% Bromphenol blue, 0.25% Xylen-Cyanol FF, 30% Glycerin |
| **Tris-borate-EDTA (TBE) buffer, 1x** | 90mM Tris, 2mM boric acid, 0.01 M EDTA, ph 8.0 |
| **1 kb DNA ladder** | Invitrogen, Carlsbad |

### Microbiology

| | |
|---|---|
| **Ampicillin solution** | 50mg/ml Ampicillin in 70% ethanol |

| | |
|---|---|
| **Freezing solution for bacteria** | 132.3 mM $KH_2PO_4$, 21mM Sodiumcitrate, 3.7mM $MgSo_4$*7 $H_2O$, 68.1mM $(NH)_2SO_4$, 459.3mM $K_2HPO_4$*3 $H_2O$, 35.2% Glycerin, autoclaved |
| **Chloramphenicol solution** | 34mg/ml Chloramphenicol in 70% ethanol |
| **Kanamycin** | 10mg/ml Kanamycin in $H_2O$ |
| **LB medium** | 10g Bacto Tryptone, 10g NaCl, 5g yeast extract, 1l Milipore water, autoclaved |
| **S.O.C. medium** | 0.5% yeast extract, 2% Bacto Tryptone, 10mM NaCl, 2.5mM KCl, 10mM MgCl, 10mM, $MgSo_4$, 20mM glucose, sterile filtered |

## Enzymes

| | |
|---|---|
| **Gateway BP Clonase II** | Invitrogen, Carlsbad |
| **Gateway LR Clonase II Plus** | Invitrogen, Carlsbad |
| **Phusion DNA Polymerase** | NEB, Frankfurt |
| **Restriction enzymes** | NEB, Frankfurt |
| **T4 DNA Ligase with 10mM ATP** | NEB, Frankfurt |
| **Taq DNA polymerase** | NEB, Frankfurt |

## Primers

| **Application** | **Primer sequence (forward, reverse)** |
|---|---|
| **Amplification of the Bar element from pN3-Bar-Gluc** | Fw TCTGACCCGTCGACGTAACCGTATTACCGCCATGC<br>Rv TACCGATTGTACAGAGCTCGGTACCAAGCTTC |
| **Amplification of the** | Fw GGGGACAAGTTTGTACAAAAAAGCAGGCTTA- |

| | |
|---|---|
| **Bar and HH element and insertion of Gateway sites** | GCGGCCGCAATAAAAT<br>Rv GGGGACAACTTTTGTATACAAAGTTGT-ATGGTGGCTGGATAAGCT |
| **Amplification of Gli1 and insertion of Gateway sites** | Fw GGGGACAACTTTGTATACAAAAGTTGTACCTCTG AGACGCCATGTTC<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTCCCT TAGGAAATGCGATCTG |
| **Amplification of TCF7 and insertion of Gateway sites** | Fw GGGGACAACTTTGTATACAAAAGTTGTAAGCTCG GATCCACTAGTAAC<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTGCCT CAGAAGCCATAGAG |
| **Amplification of dTomato** | Fw GGGGACAACTTTGTATACAAAAGTTGTAATCATTT TGGCAAAGAATTGG<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTTCTA GCTACCTAGCTAGTTTAC |
| **Amplification of WNT1 and insertion of Gateway sites** | Fw GGGGACAACTTTGTATACAAAAGTTGTACCTTCAC CATGGGGCTCT<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTTCAC AGACACTCGTGCAGTAC |
| **Amplification of WNT3 and insertion of Gateway sites** | Fw GGGGACAACTTTGTATACAAAAGTTGTAATGGAG CCCCACCTGCT<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAC TTGCAGGTGTGCACG |
| **Amplification of WNT3A and insertion of Gateway sites** | Fw GGGGACAACTTTGTATACAAAAGTTGTAATGGCC CCACTCGGATAC<br>Rv GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAC TTGCAGGTGTGCACGTC |

## Plasmids and vectors

| Plamid | Description | Source | Application |
|---|---|---|---|
| **GLI K12 (16419)** | Source of GLI 1 element for HH reporter activation | Addgene | PCR template for GLI1 overexpression entry clone |
| **pCAG-Tomato** | Plasmid encodes for fluorescent protein Tomato | M. Rosemann | PCR template for tomato entry clone |
| **pcDNA6.2PLITRHYGRO** | Backbone with hygromycin selection | C. Jäckel | Destination vector in Gateway cloning LR reactions |
| **pcDNA-Gluc3-CMVMin-Control** | Insert not responsive to Wnt or HH signaling | C. Jäckel | Control plasmid for reporter plasmids with *Gaussia* Luciferase read-out |
| **pcDNA3-HA-TCF1 (40620)** | TCF7 sequence for Wnt reporter activation | Addgene | PCR template for TCF7 overexpression entry clone |
| **pCMV(CAT)T7-SB100** | Plasmid encodes Sleeping Beauty Transposase | Addgene | Cotransfection in stable transfection of cells |
| **pENTR221-CMV** | Gateway 2 fragment cloning, first fragment, entry clone encoding the CMV promoter | C. Jäckel | Gateway cloning WNT1, WNT3 and WNT3A expressing plasmids |
| **pENTR221-CMVTO** | Gateway 2 fragment cloning, first fragment, entry clone | C. Jäckel | Gateway cloning TCF7 and GLI inducible expression vectors |

| | | | |
|---|---|---|---|
| | encoding the doxycycline inducible CMV TO promoter | | |
| **pENTR223.1-WNT3** | Canonical Wnt activator Wnt3 encoding gene | DNASU | PCR template for WNT3 overexpression entry clone |
| **pLX304-WNT3a** | Canonical Wnt activator Wnt3a encoding gene | DNASU | PCR template for WNT3A overexpression entry clone |
| **pN3-BAR-Gluc** | Reporter plasmid for canonical Wnt signaling with *Gaussia* Luciferase read-out | P. Neth | PCR template for the Bar CMV Min element for pcDNA Gluc3 Bar(pN3) |
| **Wnt1 STOP (35868)** | Canonical Wnt activator Wnt1 encoding gene | Addgene | PCR template for WNT1 overexpression entry clone |

## Recombinant proteins and pathway modulators

| | |
|---|---|
| **EGF, human** | Strathmann, Hamburg |
| **Recombinant human Wnt-3a protein** | R&D, Minneapolis |
| **Lithium chloride** | Sigma-Aldrich, St. Louis |

## Kits

| | |
|---|---|
| **Endofree Plasmid Maxi Kit** | Qiagen, Hilden |
| **BioLux *Gaussia* Luciferase Assay Kit** | NEB, Frankfurt |
| **Innuprep Plasmid Midi Direct Kit** | Analytik Jena, Jena |

| | |
|---|---|
| **Innuprep Plasmid Mini Kit** | Analytik Jena, Jena |
| **Lipofectamine® 2000 Transfection Reagent** | ThermoFisher, Waltham |
| **Neon™ Transfection System 10µL Kit** | Invitrogen, Carlsbad |
| **QIAquick Gel Extraction Kit** | Qiagen, Hilden |

## Laboratory equipment

| | |
|---|---|
| **$CO_2$ incubator New Brunswick™ Galaxy® 48 R** | Eppendorf, Hamburg |
| **Fluorescence activated cell scanner FACSCalibur** | BD Biosciences, San Jose |
| **Heating stage for migration assay** | Ibidi, Martinsried |
| **Inverted fluorescence microscope DMIL** | Leica, Wetzlar |
| **Nano Drop ND-1000 Spectrophotometer** | PEQLAB Biotechnologie GmbH, Erlangen |
| **Neon® Transfection System** | ThermoFisher, Waltham |
| **PCR machine Mastercycler pro** | Eppendorf, Hamburg |
| **Photo Camera for Leica DMIL** | Jenoptik, Jena |
| **Photometer Lumat 9507** | Berthold, Bad Wildbad |

## Consumables

| | |
|---|---|
| **FACS tubes** | BD Bioscience, San Jose |
| **Tubes, 5ml 75x12mm, PS (for luminometer)** | Sarstedt, Nümbrecht |
| **PCR tubes Eppendorf 0.2ml** | Eppendorf, Hamburg |
| **µ-Slide Chemotaxis 3D, ibiTreat** | Ibidi, Martinsried |

## Software

| Name | Application | Source |
|---|---|---|
| **Prism** | Statistical analysis and generation of graphs | GraphPad Software, La Jolla |
| **ImageJ** | Analysis of migration assays and microscopy pictures | National Institute of Health, USA |
| **CellQuest** | Analysis of FACS data | BD Bioscience, San Jose |
| **Chemotaxis and Migration Tool – ImageJ Plugin** | Analysis of migration assays | Ibidi, Martinsried |
| **Clone Manager Professional 9.2** | Planning cloning | Sci-Ed, Denver |
| **pDraw32** | Generation of plasmid maps | Kjeld Olesen |
| **ProgRes Capture software** | Taking pictures | Jenoptik, Jena |

# Methods

## Cell culture

### General cell culture

Cells were cultured at 37°C and 5% $CO_2$ in an incubator. Cell culture was conducted in a laminar flow hood to prevent contamination. All reagents were warmed to room temperature prior to use.

For subculture, adherent cells were washed with PBS, detached using Trypsin-EDTA solution until detachment of the cells was visible under the microscope, and then the reaction was stopped by adding double the volume of a medium containing 10% FCS. After pelleting cells by centrifugation for 3min at 200g, they were resuspended in fresh medium and seeded into an appropriate cell culture vessel.

### Freezing and thawing of cells

Cells were frozen in cryo-tubes with a freezing medium, containing the following: 55% of their respective medium, 35% FCS, 10% DMSO. Subsequently, they were cooled to -80°C for at least 24h in an isopropanol container. For long-time storage, cells were put into the liquid nitrogen storage.

To thaw cells, the frozen cell suspensions were rapidly warmed and were immediately transferred into a cell culture vessel filled with medium containing 10% FCS to dilute DMSO to a non-toxic concentration. The medium was changed within 24h to remove DMSO completely.

### Counting cells

To determine the number of cells per ml, a Neubauer counting chamber was used. An aliquot of the cell suspension was mixed 1:2 with Trypan blue solution (0.4%) in order to differentiate viable from dead cells. Then, the chamber was filled with the suspension and cells in all four corner squares were counted. The cell number was calculated, see equation 1.

$$average\ cell\ number\ per\ square\ * 10^4 * dilution = cell\ number\ /ml$$

**Equation 1 Calculating number of cells using a Neubauer counting chamber**

### Cell culture for primary mesenchymal stem cells

Primary mesenchymal stem cells (MSC) were isolated form bone marrow aspirates taken in AKB Gauting. Isolation, expansion and differentiation assay of primary MSC (called hbMSCAp172_1) was conducted by the company Apceth GmbH & Co. Primary MSCs used in this thesis were all retrieved from one patient. For migration experiments, MSCs were cultured in medium on DMEM basis provided by the company Apceth GmbH & Co, the exact constitution being propriatory.
For all other experiments, except for the migration assays, a medium consisting of DMEM, 10% FCS, 1% PS, 2IE/ml Na-Heparin and 5% platelets was used.
The medium was changed every two to three days.

## Molecular biology

### Preparation of agar plates

LB medium was prepared with mixing 10g of tryptone, 5g of yeast extract, 10g of NaCl in 950ml deionized water, adding 1N NaOH and 15g/L agar before autoclaving. When required, appropriate selection antibiotics were added after cooling (final concentration of Ampicillin 100µg/ml or Kanamycin 50µg/ml). The liquid agar was poured into petri dishes, let hardened and then the agar plates were stored in sealed plastic bags at 4ºC.

### Freezing and thawing of bacteria

Bacterial glycerol stocks were established for long-time storage of *E.coli* strains. To this end, 900µl of an overnight culture were mixed with 100µl freezing solution for bacteria (10x) and frozen at -80ºC. For extraction of DNA, a liquid culture was inoculated with a small amount of frozen bacteria and a Midi or Maxi Prep was performed.

**Test digestion of DNA**

DNA produced from Mini preps was digested in order to send the most promising clone for sequencing. Suitable test digests were identified using CloneManager Software (Sci-Ed, Denver) preferably finding enzymes cutting once in the backbone and once in the insert. We used 1µl enzyme per 1µg DNA per hour, but maximal 10% of enzyme in the mix in total. DNA concentrations after Mini prep were estimated 30 to 100ng/µl. The double digest finder from New England Biolabs (www.neb.com) was used to identify the most suitable puffer and temperature.

**Separation of DNA fragments by gel electrophoresis**

Agarose gel electrophoresis was used to separate DNA fragments for analytical or preparative purposes. DNA fragments were separated by gel-electrophoresis at 160 Volt. Gel containing a suitable percentage of agarose was chosen, for example 2% or 0.6% for fragments under 800 or over a 1000 base pairs, respectively.

**Gel extraction**

The QIAquick Gel Extraction Kit (Qiagen) was used according to the manufacturer's protocol.

**Determination of DNA concentrations**

Nanodrop photometer was used to photometrically determine the amount of DNA in a sample. Nanodrop was initialized with 2µl RNAse free water and blanked with 2µl of the substance in which DNA was diluted.

**Transformation of competent E. Coli Mach1**

*E.coli* Mach1 were stored at -80°C and thawed on ice. After adding the DNA to the tube and gently mixing, incubation on ice was performed for 5 to 30min. After heat-shocking at 42°C for 30sec, the tube was immediately transferred back on ice. Then, 250µl room temperatured S.O.C. medium was added, before shaking the tube horizontally for 1h at 37°C with 500rpm. The content of the tube was spread on a pre-warmed selective plate and incubated overnight at 37°C.

**Isolation and analysis of plasmid DNA from transformed bacteria**

Flasks with 50ml LB medium and the appropriate selection antibiotic were inoculated with *E.coli* bacteria from the glycerine stock (-80°C) or the backup plate

(4°C). See table 3 for the final concentration of the respective selection antibiotic in the LB medium. After incubation overnight on a warmed shaking device, the plasmids were isolated from *E.coli* bacteria using Qiagen Maxi or Midi Prep Kit. The EndoFree Plasmid MaxiKit was used according to the Protocol 'Plasmid or Cosmid DNA Purification'. DNA was diluted in 250-400µl buffer TE.

**Table 3 Final concentration of selection antibiotic in LB medium used for inoculating *E.coli***

| Selection antibiotic | Concentration |
|---|---|
| **Chloramphenicol** | 25µg/ml |
| **Ampicillin** | 120µg/ml |
| **Kanamycin** | 50µg/ml |
| **Spectinomycin** | 50µg/ml |

**Polymerase chain reaction (PCR)**

PCR is used to amplify DNA. Suitable primers were ordered from Invitrogen LifeTechnologies and dissolved in the indicated amount of TE. Annealing temperature and elongation time were set for every PCR individually. See table 4 and 5 for standard PCR settings.

**Table 4 Standard polymerase chain reaction (PCR) settings**

| **Standard settings PCR** | |
|---|---|
| **98°C 3min** | Initialization step |
| **98°C 30sec** | Denaturation step |
| **Temperature dependent on primer 30sec** | Annealing step |
| **72°C and seconds per cycle dependent on length of the product** | Extension/elongation step |
| **72°C 10min** | Final elongation |
| **10°C** | Final hold |

**Table 5 Standard PCR mix**

| **Standard PCR mastermix (per PCR tube 25µl)** | |
|---|---|
| **1:5** | 5x buffer Phusion HF Reaction Buffer |
| **200nM** | dNTPs |
| **400nM** | Forward primer |
| **400nM** | Reverse primer |
| **500ng** | Template DNA |
| **4.5 units** | Phusion HF Polymerase |
| **Add up to 225µl** | Aqua ad iniectabilia Braun |

*If the PCR did not yield the expected bands, test PCRs were conducted adding 1-3% DMSO. For further cloning steps 225µl total volume were suitable.*

**Sequencing of DNA**

Samples were sent to GATC Biotech company for sequencing using Sanger's method.

## Cloning strategies & Plasmids

Cloning was conducted in cooperation with Carsten Jäckel generally as described (Jackel et al., 2016).

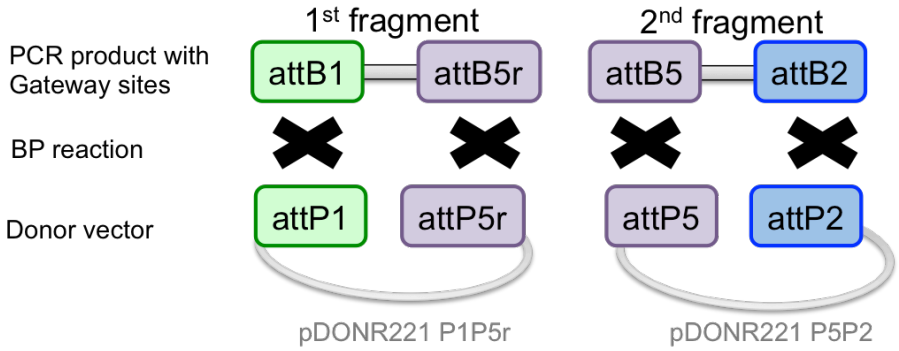
### Gateway Cloning Technology

Invitrogen's Gateway Technology allows rapid and highly efficient transfers of heterologous DNA sequences into multiple vector systems. It is a universal cloning system based on the bacteriophage lambda site-specific recombination system which facilitates the integration of lambda into the *E.coli* chromosome.

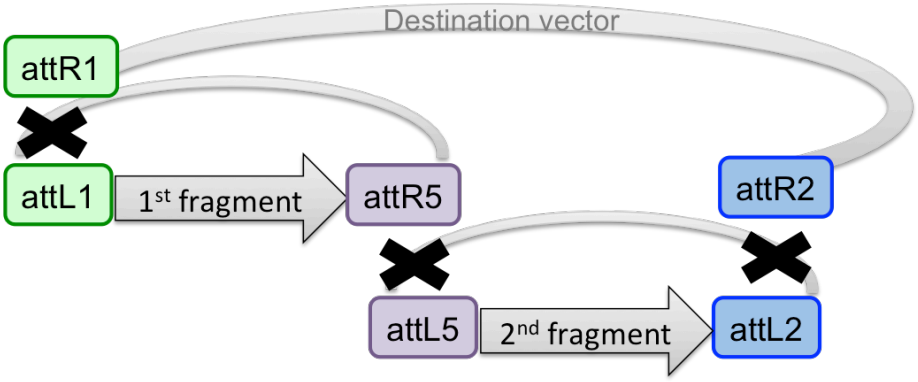
Gateway Technology enables efficient transfer of DNA-fragments between plasmids using a set of recombinant sequences and enzyme mixes. The general principle is as follows. First, a DNA fragment of interest is amplified using PCR with Gateway recombinant sites added to the primers. After separation of the DNA fragments by gel electrophoresis and gel extraction, 150ng of the DNA was used to perform the BP reaction. Next, the BP reaction was performed overnight, stopped and the preparation was transformed into *E.coli* which were then plated onto a LB plate. The next day, colonies were picked and Mini Preps were inoculated, also marking the clones on a backup LB plate. After DNA preparation, the DNA was digested and sent to the company GATC for sequencing. Subsequent LR reactions allow a combination of up to three different entry clones in one backbone yielding the final plasmid. After LR reactions, the preparation was again used to transform *E.coli* and DNA extraction followed. Cloning success was confirmed by test digests and sequencing.

## 2-fragment Gateway cloning

**1. PCR:** Amplification of DNA of interest with specific attB-containing PCR primers to insert Gateway sites.
**2. BP reaction**: Generation of entry clones with attB-flanked PCR fragments and the appropriate donor vector.




**3. LR reaction**: Recombination of entry clones with a destination vector (with attR1 and attR2 sites) to generate an expression vector.




**4. Expression vector**

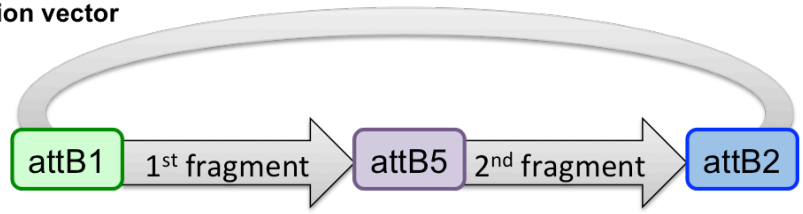



**Figure 6 Schematic overview of 2-fragment multisite Gateway cloning**

*Every Gateway cloning process includes PCR, BP and LR reaction in order to obtain the final vector.*

***BP Reaction***

In a BP reaction, an entry clone is produced from an attB flanked PCR product and a donor vector. Depending on the experimental set-up, the suitable attB flanks were added to the PCR product and the correct donor vector was chosen. For example, in a 2-fragment reaction, the PCR product of the first fragment was attB1 and attB5r flanked and the pDONR221 P1P5r. The PCR product of the second fragment was flanked by attB5 and attB2 and pDONR221 P5P2 was used (Figure 6). The following components were pipetted together in a 1.5ml microcentrifuge tube: 150ng attB PCR product (1-7µl), 150ng pDONR vector and 1x TE buffer (filled up to 8µl). The enzyme BP clonase is temperature sensitive, after removing it from the -20°C storage, it was carefully shaken and centrifuged; a volume of 2µl was added to the microcentrifuge tube. After briefly vortexing and centrifuging, the reaction was incubated overnight at room temperature. The reaction was stopped by adding 1µl of Proteinase K and incubating at 37°C for 10min. Afterwards, Mach1 *E.coli* transformation followed, for vectors cloned within the scope of this thesis, with bacterial kanamycin selection.

***LR Reaction***

In a LR reaction, an expression vector is produced from one of up to four entry clones and a destination vector. The following components were pipetted together in a 1.5ml microcentrifuge tube: 40fmol destination vector, 20fmol per entry clone and 1x TE buffer (filled up to 8µl). The following calculation is used to determine the needed amount of plasmid (Equation 2).

$$(x\ fmol) * (y\ bp) * \left(\frac{660fg}{fmol}\right) * \left(\frac{1ng}{10^6 fg}\right) = z\ ng$$

***Equation 2 Coverting Femtomoles (fmol) to Nanograms (ng)***

*x: amount of fmol one would like to use; y: amount of plasmid's base pairs (bp); z: amount of plasmid needed for the LR reaction in ng.*

All LR reactions relevant for thesis were 2-fragment reactions. In this case, the entry clone for the first fragment contained attL1 and attR5 sites and the entry clone for the second fragment contained attL5 and attL2. Destination vectors were chosen according to the selection antibiotic for stably transfected cells, enabling cotransfections.

The enzyme LR Clonase II was vortexed briefly and 2µl were added to the microcentrifuge tube. After an overnight incubation at room temperature, the LR reaction was stopped with 1µl Proteinase K and incubated at 37°C for 10min. Afterwards Mach1 *E.coli* transformation followed, for our cloned vectors with bacterial ampicillin selection.

**Tetracycline-controlled transcriptional activation**

Tetracycline-controlled transcriptional activation was used for inducible gene expression where transcription is regulated through addition of the antibiotic tetracycline or its derivatives, for example doxycycline.

For work in scope of this thesis, reporter plasmids that carry the TetRepressor and inducible expression constructs were tet-operated. After cotransfection of the reporter and the expression plasmid, doxycycline was added to activate gene expression. The system enabled the effects of specific genes stimulated with doxycycline to be compared to unstimulated cells.

**Reporter plasmids & other Wnt signaling**

***pcDNA-Gluc3-CMVMin-Bar***

The TCF/LEF-reporter-plasmid measures β-catenin dependent expression in transfected cells. Canonical Wnt signaling is β-catenin dependent. Activity is measured using *Gaussia* luciferase reporter activity secreted into the growth media (Jackel et al., 2016). *Gaussia* luciferase is derived from the marine copepod *Gaussia* princeps; the luciferase catalyzes the oxidation of the substrate coelenterazine in a reaction that produces light and can be measured from the cells' supernatant with a luminometer.

In our reporter system, *Gaussia* luciferase is expressed and secreted when β-catenin associated with TCF/LEF transcription factor after translocation into the nucleus. The Bar (β-catenin activated reporter) element was located directly in front of a minimal CMV promoter and consisted of 12 TCF/LEF responsive elements (AGATCAAAGG) divided by spacer sequences of 5 bases (Figure 7, (Jackel et al., 2016).

The plasmid's backbone was created by digesting the plasmid pcDNAGluc3_Rantes (available in our working group) with the enzymes BsrGI and XhoI. The Bar element was amplified from the plasmid pN3-BAR-Gluc (Peter Neth) using PCR. As the restriction sites from the enzymes SgrD1 and Xho1 were compatible, ligation of the backbone with the Bar element was possible. For ligation, 10x Buffer for T4 DNA Ligase with 10mM ATP and T4 DNA Ligase, and 1:3 molar ratio of backbone to insert were used.

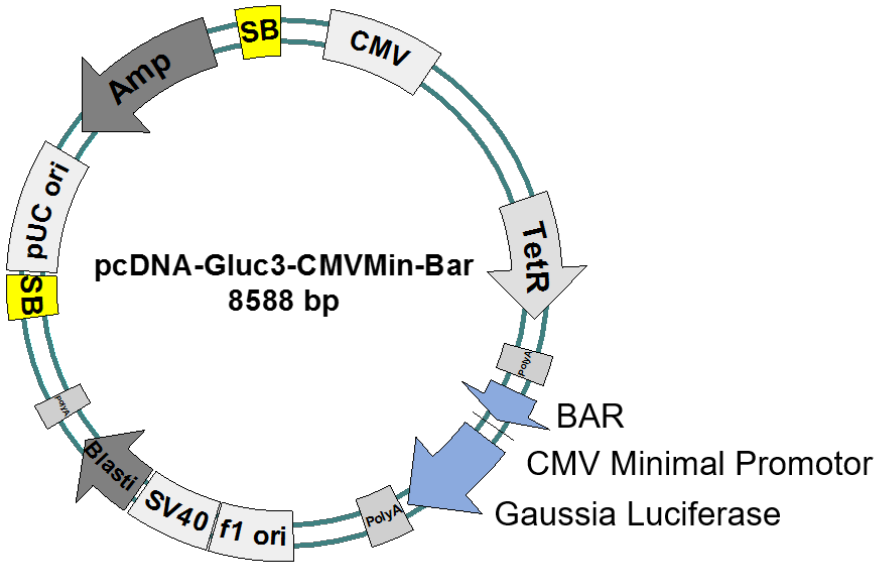



**Figure 7 pcDNA-Gluc3-CMVMin-Bar, a canonical Wnt reporter plasmid**

*In this canonical Wnt signaling reporter plasmid, the TCF/LEF responsive luciferase construct encodes the Gaussia luciferase reporter gene under the control of a CMV Minimal Promoter with Wnt responsive elements. Bar (β-catenin activated reporter) element uses 12 TCF/LEF binding sites; Min CMV: CMV minimal promoter, Gaussia Luciferase: CDS of the Gaussia Lucifrease; f1 ori: origin of replication; SV40: SV40 promoter; Blasti: Blasticidin resistance gene; TetR: TetRepressor; SB: Inverted terminal repeats for Sleeping Beauty 100 transposase.*

***pcDNA 6.2Hygro CMV TO TCF7***

This plasmid served as a positive control for the Wnt reporter plasmids, as it is an inducible expression construct of the transcription factor TCF7 (former name TCF-1). When cotransfected with our reporter plasmids, expressing the TetRepressor, there is no expression of TCF7. Derepression through doxycycline leads to the induced expression.

The plasmid was created using Gateway Cloning Technology. The Addgene plasmid 40620: pcDNA3-HA-TCF1 was used as a PCR template. In a 2-fragment multisite Gateway cloning reaction the entry clones CMV-TO and TCF7 were united in a destination vector expressing the hygromycin selection gene (Figure 8).

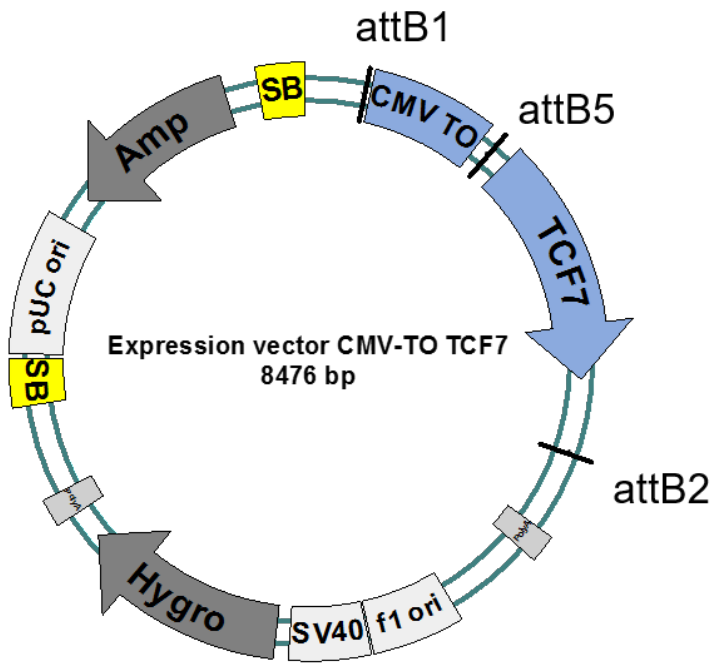



**Figure 8 Expression vector CMV-TO TCF7, an inducible Wnt signaling activator**

*The expression vector CMV-TO TCF7 is a doxycycline inducible expression construct, serving as a positive control for the Wnt reporter plasmids. CMV TO: CMV promoter tet-operated; f1 ori: origin of replication; SV40 promoter; Hygro: Hygromycin resistance gene; SB: Inverted terminal repeats for Sleeping Beauty 100 transposase; Amp: Ampicillin resistance gene for bacterial selection. Gateway cloning sites attB1, attB5 and attB2.*

***Canonical Wnt stimulating plasmids***

In order to activate canonical Wnt signaling; WNT1, WNT3 and WNT3A gene sequences were obtained by PCR from the plasmids Wnt1 STOP, pENTR223.1-WNT3 and pLX304-WNT3a respectively.

The respective PCR product was combined with the donor vector pDONR221 P5P2 in a BP Gateway cloning reaction yielding a suitable 2nd fragment entry clone for 2-fragment cloning. In LR reactions the entry clone of the constitutively active CMV Promoter was combined with the respective WNT entry clones in a destination vector with Hygromycin selection (Figure 9).

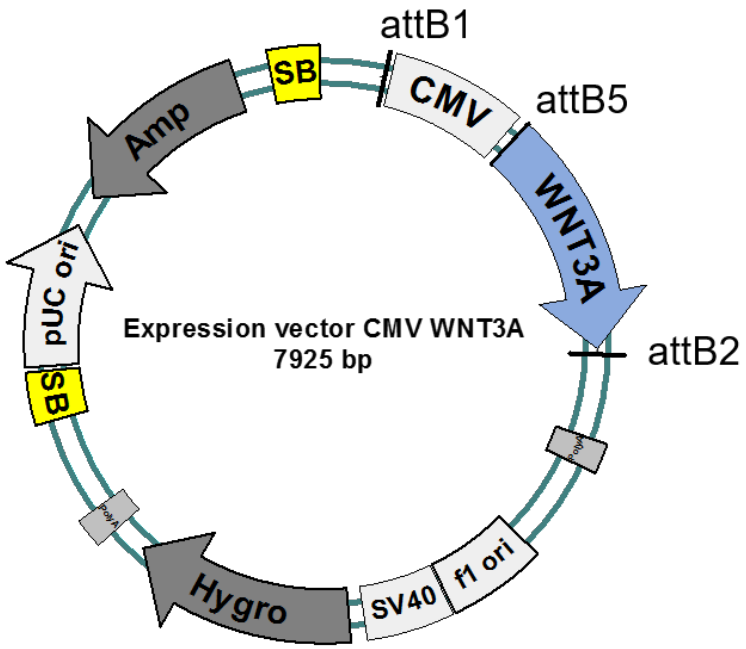



**Figure 9 Expression vector CMV WNT3A, a plasmid constitutively expressing the WNT3A gene**

*The expression vector CMV WNT3A constitutively expresses the WNT3A gene driven by a CMV promoter. The expression vectors CMV WNT1 and WNT3 contain the respective insert instead of WNT3A. f1 ori: origin of replication; SV40 promoter; Hygro: Hygromycin resistance gene for selection of transfected cells; SB: Inverted terminal repeats for Sleeping Beauty 100 transposase; Amp: Ampicillin resistance gene for bacterial selection. Gateway cloning sites attB1, attB5 and attB2.*

***pcDNA6.2ITRHygro-BarCMVMin-Tomato (Bar-Tomato)***

This plasmid is a TCF/LEF-reporter-plasmid, β-catenin dependent activation leads to increased expression of the fluorescent protein Tomato in transfected cells. Peak absorption and emission are at 554 nm and 581 nm respectively. Tomato is a tandem dimer, equally photostable as mCherry fluorescent protein, but twice the molecular weight of mCherry.

The reporter plasmid was generated via Gateway Multisite Cloning inserting two fragments, namely pENTR221-Bar CMV Min and pENTR221-Tomato.

The entry clone pENTR221-Bar CMV Min was created by amplifying the Bar element from the plasmid pcDNA-Gluc3-Bar(pN3), and by adding the Gateway sites using PCR. Using gel electrophoresis the correct PCR product was identified as a 599bp band, that was then cut out and extracted from the gel. In the BP reaction, the PCR amplicon was cloned into the pDONR221 P1P5r backbone.

The plasmid pCAG-tomato was used as a PCR template for generating pENTR221-tomato. Using gel electrophoresis, the correct PCR product was identified as a 1523bp band, that was cut out and extracted from the gel. In the BP reaction, the PCR amplicon was cloned into the backbone pDONR221 P5P2.

In an LR reaction the first fragment entry clone Bar, and the second fragment entry clone Tomato, were put together into the destination vector pcDNA6.2.ITRHygro, a vector expressing SB ITRs and a hygromycin selection gene (Figure 10).

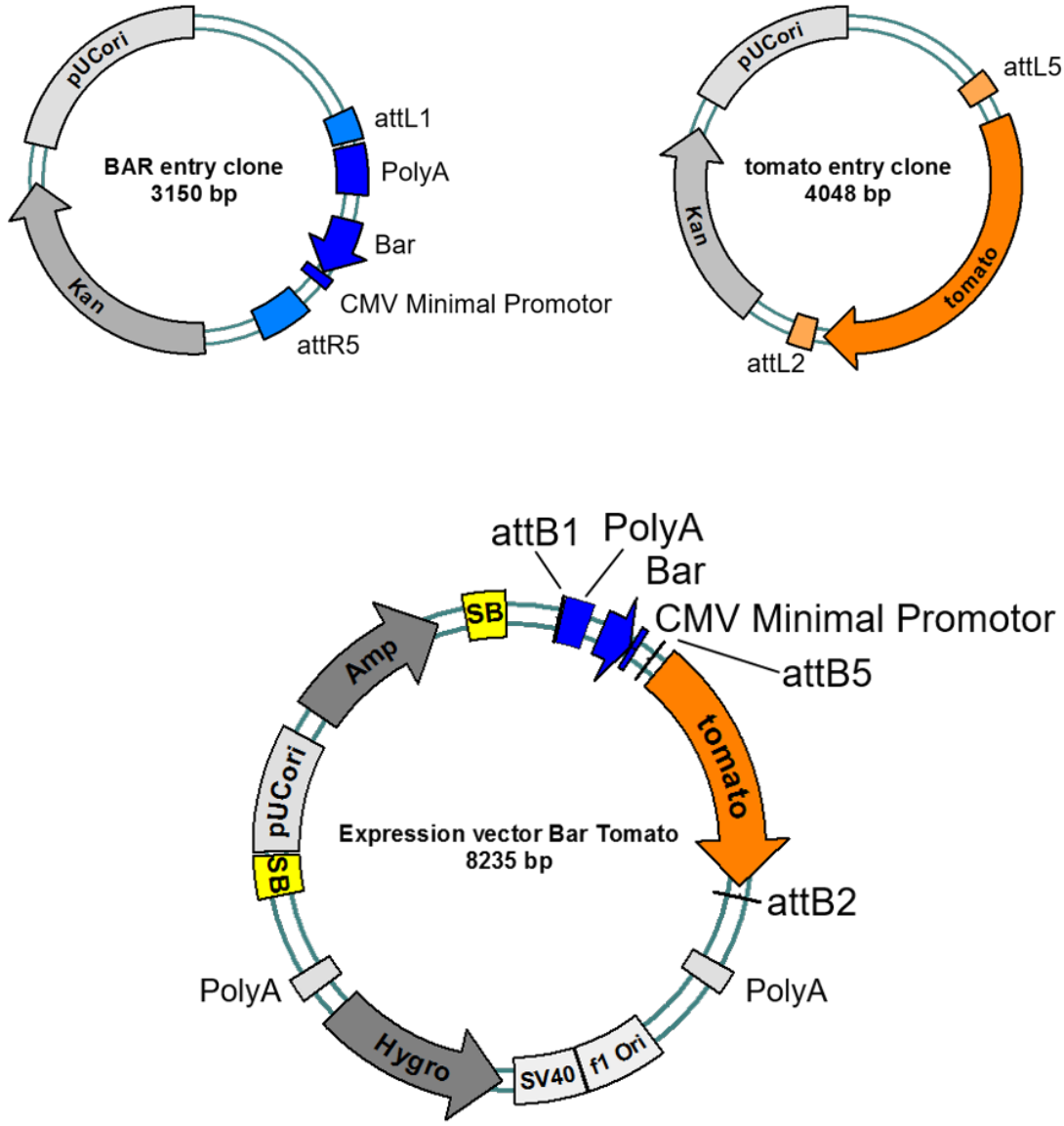



**Figure 10 Expression vector Bar-Tomato was created using the Bar and Tomato entry clone**

*The expression vector Bar Tomato was created through Multisite Gateway Cloning. In a 2-fragment reaction the entry clones Bar and Tomato were united in the destination vector pcDNA6.2.ITRHygro. The expression vector Bar Tomato has Ampicillin (Amp) selection for bacteria and Hygromycin (Hygro) selection for transfected cells. Sleeping Beauty inverted terminal repeats (SB) enable an efficient transfection. Gateway cloning sites are marked with attB1, attB5 and attB2.*

**Reporter plasmids & other Hedgehog signaling**

***pcDNA-Gluc3-CMVMin-HH(Hedgehog)***

The Gli responsive reporter plasmid is a reporter used for detecting Hedgehog signaling. The reporter plasmid possesses eight Gli binding sites with the sequence GACCACCCA. Gli activation leads to *Gaussia* Luciferase production and can be measured from the supernatant in the *Gaussia* Luciferase assay (Figure 11).

The Gli responsive element was originally cloned from the commercially available plasmid Cignal GLI Reporter (luc) Kit: CCS-6030L (Qiagen), subcloned and, finally, classically cloned into our reporter plasmid (Jackel et al., 2016).

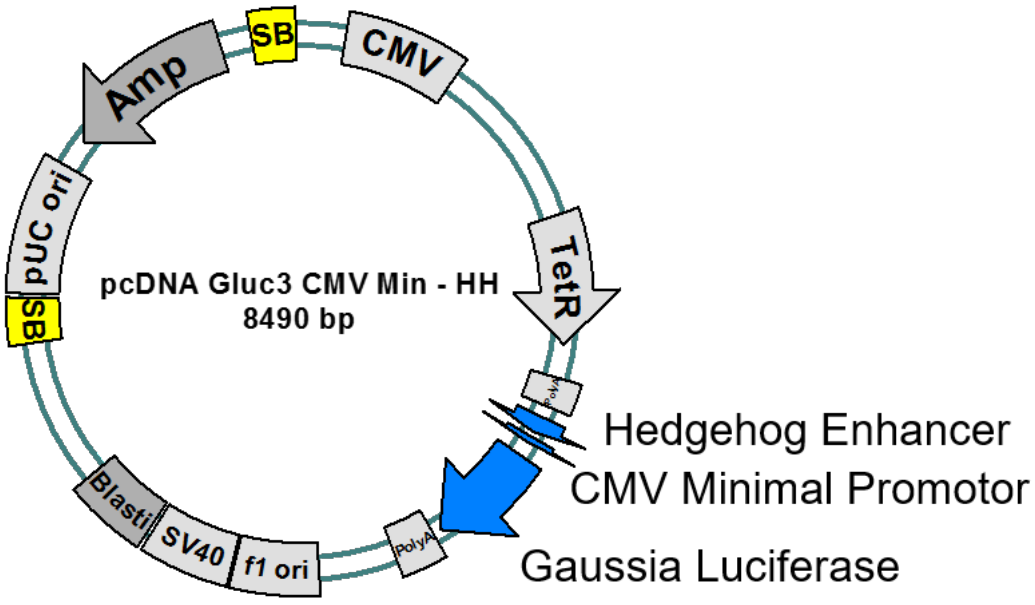



**Figure 11 pcDNA-Gluc3-CMVMin-HH, a reporter plasmid for Hedgehog signaling**

*In this HH reporter plasmid, the Gli responsive luciferase construct encodes the Gaussia luciferase reporter gene under the control of a CMV Minimal Promoter. Hedgehog responsive via eight Gli binding sites; CMV: CMV promoter; Gaussia Luciferase: CDS of the Gaussia Lucifrease; f1 ori: origin of replication; SV40: SV40 promoter; Blasti: Blasticidin resistance gene; TetR: TetRepressor; SB: Inverted terminal repeats for Sleeping Beauty 100 transposase*

***pcDNA6.2PLITRHygro_Dest-CMV-TO-Gli1***

This plasmid served as a positive control for experiments studying Hedgehog signaling. It allows inducible expression of the transcription factor human glioma-associated protein1 (Gli1). When cotransfected with reporter plasmids expressing the TetRepressor, there is no expression. Derepression through doxycycline leads to induced expression of Gli1.

To obtain the Gli1 entry clone, the plasmid 16419: GLI K12 (Addgene) was used as a PCR template. In a 2-fragment multisite Gateway cloning reaction, the entry clones CMV-TO (Carsten Jäckel) and Gli1 were united a destination vector expressing the hygromycin selection gene (Figure 12).

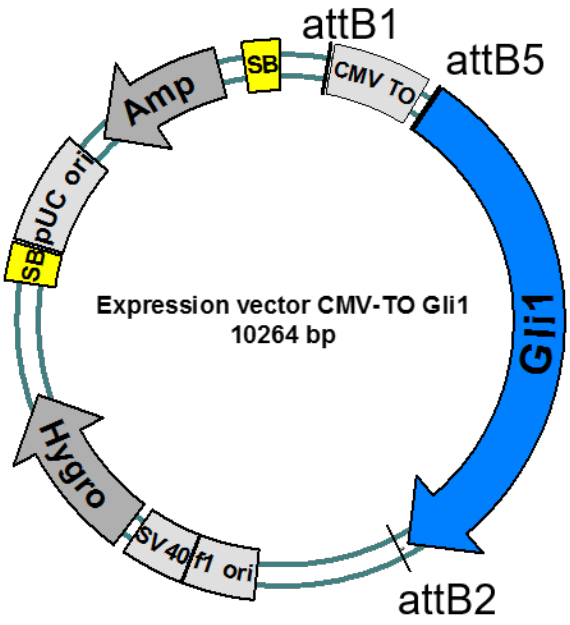



**Figure 12 Expression vector CMVTO-Gli1, an inducible Hedgehog signaling expression construct**

*The expression vector CMV-TO Gli1 is a doxycycline inducible expression construct, serving as a positive control for the Hedgehog (HH) reporter plasmids. CMV TO: CMV promoter tet-operated; f1 ori: origin of replication; SV40 promoter; Hygro: Hygromycin resistance gene; SB: Inverted terminal repeats for Sleeping Beauty 100 transposase; Amp: Ampicillin resistance gene for bacterial selection. Gateway sites attB1, attB5 and attB2.*

**Control reporter plasmid**

pcDNA-Gluc3-CMVMin-Control, a 8435bp negative control plasmid was cloned as described (Jackel et al., 2016). The insert does not contain pathway specific responsive elements but was otherwise identical in construction to the Wnt or HH reporter plasmids with *Gaussia* Luciferase. Therefore, it was used as a control to differentiate if activation of the promoter and enhanced *Gaussia* Luciferase activity were an unspecific artifact or due to specific activation of the pathway responsive element.

## Transient and stable transfection of cells

**Lipofectamine2000**

Transient transfection of cells was conducted using Lipofectamine2000. Transfection with Lipofectamine2000 is based on the principle of micelle formation through the interaction between the cationic lipofectamine reagent and negatively charged nucleic acid. The complexes are taken up by the cells by endocytosis. DNA and lipofectamine concentration per well was established for each cell line. In order to transiently transfect L87 (iMSC) or Hek293 cells in a 96-Well plate, 0.2µl lipofectamine and 200ng DNA were diluted in 25µl Opti-MEM medium respectively. After a 5min incubation, DNA and lipofectamine were mixed and incubated 20min incubation at room temperature. Then, the mix was added to the 75% confluent cells which were seeded in 50µl medium with FCS. After 6-8h the medium was changed as lipofectamine also showed a toxic effect on cells. The next day the medium was changed again to stimulation medium.

**Electroporation/Nucleofection**

The mechanism of electroporation is not well understood. The current thought is that it creates small pores in the plasma membrane, allowing large molecules, like DNA, to enter the cytosol through diffusion.

Therefore, 70-90% confluent cells were detached and counted. The needed amount of cells was put into a 1.5ml centrifuge tube and centrifuged for 1min at 7000xg. The pellet was resuspended in 10µl Puffer R and the plasmids, maximal 3µg in total per electroporation, were added. For stable transfections usually 1µg pCMV(CAT)T7-SB100 and 2µg of the respective plasmid were transfected. The established

transfection conditions for the cell line were set on the screen and 3ml Puffer E were added to the container. Neon tips 10µl were used according to the manufacturer's instructions and regenerated as described by Brees and Fransen (Brees & Fransen, 2014). Transfected cells were seeded in 25cm$^2$ flasks with 5ml medium. Later, 24h-48h after the transfection the medium was changed and the respective selective antibiotic for the plasmid was added. The concentration of the selective antibiotic was dependent on the type of antibiotic and the cell line. The lowest concentration of antibiotic was used where all not transfected cells died within 10 days for Blasticidin and Zeocin, and 14 days for Hygromycin. For primary cells, antibiotic concentrations were gradually raised to the determined final concentration.
Neon electroporation transfection protocols were optimized for every cell line individually (Table 6 and 7).

**Table 6 Transfection protocols using Neon electroporator**

| Cell line | Voltage | Width | Pulses | Cells per 10µl Puffer R |
|---|---|---|---|---|
| **hbMSC** | 1050V | 30ms | 2 | $1*10^6$ |
| **Hek293** | 950V | 30ms | 1 | $0.5*10^6$ |
| **L87 (iMSC)** | 900V | 30ms | 1 | $0,5*10^6$ |
| **V54-2 (iMSC)** | 900V | 30ms | 1 | $0,5*10^6$ |

**Table 7 Selection antibiotic concentrations for primary hbMSCs and immortalized cell lines**

| Cell line | Blasticidin (µg/ml) | Hygromycin (µg/ml) | Zeocin (µg/ml) |
|---|---|---|---|
| **hbMSC** | 2 | 40 | |
| **Hek293** | 8 | 150 | 400 |
| **L87 (iMSC)** | 6 | 200 | |
| **V54-2 (iMSC)** | 10 | 250 | |

**Stable transfection using Sleeping Beauty transposase**

Transposons, discovered in the 1940s by Barbara McClintock, are DNA segments that have the distinctive ability to move and replicate within genomes. The Sleeping Beauty (SB) transposon system is a non-viral gene transfer technology that uses a synthetic DNA transposon designed to introduce precisely defined DNA sequences almost randomly into the chromosomes of vertebrates. SB transposase inserts a transposon into a TA dinucleotide base pair in a recipient DNA sequence, resulting in a near-random integration profile. It is a reconstruction of an active vertebrate element from “dead” transposon fossils found in fish genomes. The gene of interest for integration into a genome has to be flanked by inverted terminal repeats (ITR) and the resulting plasmid has to be cotransfected with a transposase source, in scope of this thesis pCMV(CAT)T7-SB100 was used (Figure 13).

Cells were stably transfected with Neon electroporator utilizing the respective plasmid with ITRs and 1µg of the plasmid pCMV(CAT)T7-SB100, Addgene plasmid # 34879.

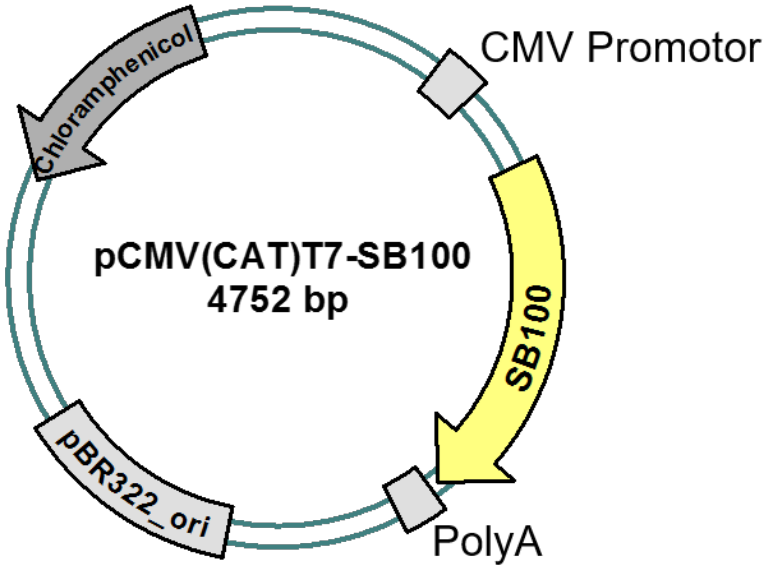



**Figure 13 Plasmid used as transposase source pCMV(CAT)T7-SB100**

*This plasmid encodes the gene of the Sleeping Beauty 100 (SB100) Transposase under the control of a CMV promoter. Chloramphenicol resistance gene for bacterial selection and a bacterial origin of replication (pBR322_ori) are present.*

## In vitro experiments

### Migration Assay

In order to analyze motility and chemotaxis of primary mesenchymal stem cells ibidi® µ-slide Chemotaxis3D chambers and protocols were used.

In this assay, $0.3x10^6$ cells/ml were embedded in a collagen I matrix applied to the provided channel. Reservoirs were filled with HUH7 conditioned medium and control medium, creating a gradient. See figure 14 for the experimental set-up. After taking a picture every 15min for 18h, cell paths of 25 randomly selected cells were tracked manually using the ImageJ Manual Tracking plug in. Analysis was conducted using the Manual Tracking and Chemotaxis Tool plug in. Migration assays were conducted in cooperation with Alexandra Wechselberger.

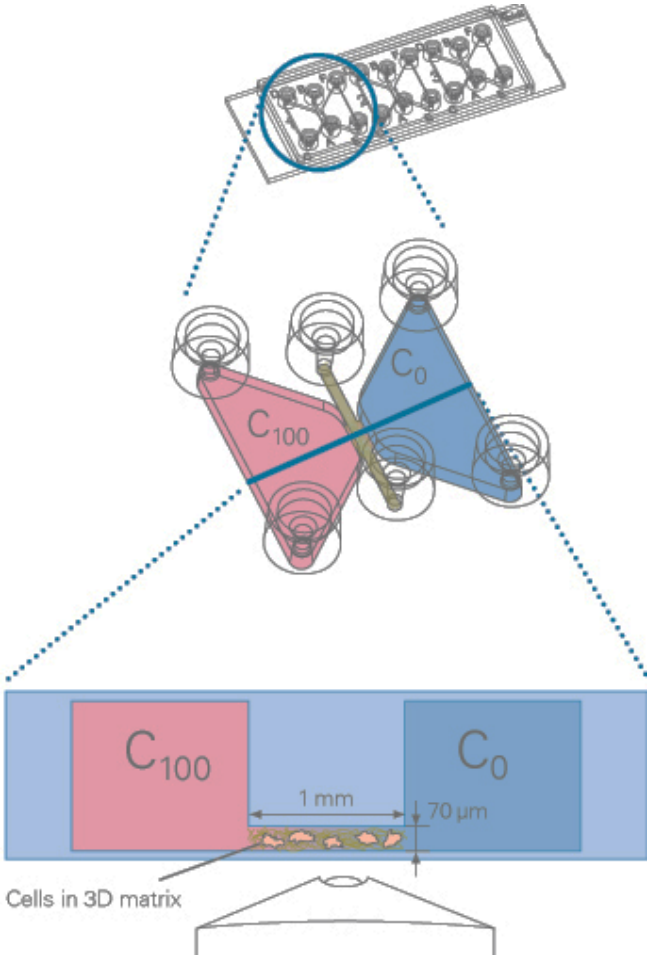



**Figure 14 Experimental set-up of a migration assay using *ibidi® µ-slide Chemotaxis3D slides***

*The gradient between the tumor conditioned medium ($C_{100}$: concentration of chemokines 100%) and the control medium ($C_0$: concentration of chemokines 0%) is shown. Cells embedded in a 3D matrix are exposed to the gradient and cell migration is observed through a microscope. (Picture modified from ibidi.com)*

***Assay medium and collagen gel***

For the migration of primary cells, DMEM assay medium (DMEM, 1% BSA, 15mM HEPES) and DMEM 1mg/ml collagen were used. The gel with the collagen and a suspension of $0.6*10^6$ cells in 12.5µl were carefully mixed 1:1 on ice and then 6µl were pipetted per slot.

***HUH7 supernatant***

HUH7 supernatant is an established MSC chemoattractant source in our lab. To generate HUH7 cell supernatant, the cells were plated in a 10cm cell culture dish and grown until 100% confluency was reached. Then, the medium was changed to assay medium. After 48h the supernatant had been removed and centrifuged at 200xg for 5 min. Aliquots of 250µl in 1.5 microcentrifuge tubes were stored at -20°C.

***Microscope settings***

Chemotaxis was monitored by time-lapse microscopy over an 18h period on a Leica DM IL microscope on a heated stage at 37°C. Pictures were taken in each experiment consistently every 15min with a Jenoptik ProgRes CCD camera.

***Chemotaxis parameters***

To quantify chemotaxis and migration, several values can be generated using the Chemotaxis and Migration Software Tool in the computer program ImageJ (National Institute of Health, USA). Around 25 cells were randomly selected and manually tracked (Figure 15).

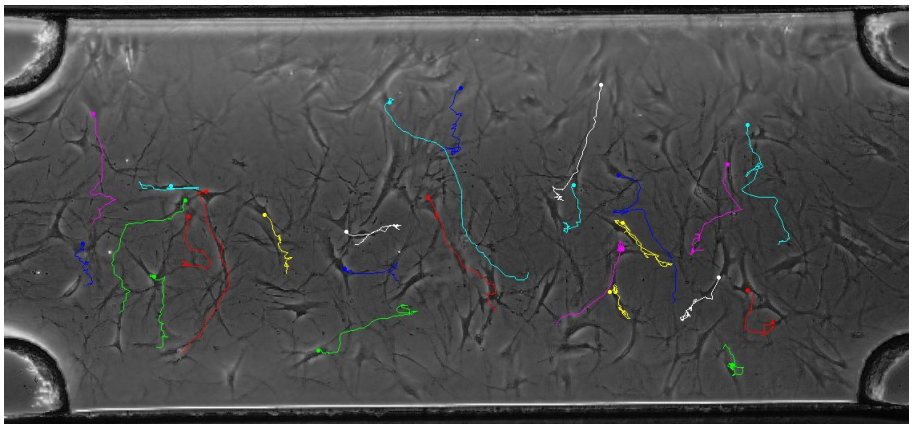


**Figure 15 Microscopic picture of an *ibidi® µ-slide Chemotaxis 3D with primary MSCs embedded in a 3D collagen I matrix with every colored track indicating migration of one cell***

*For an 18h migration 72 pictures are taken and manually tracked using ImageJ software. Each colored track represents the migration of one cell during the entire experiment.*

Center of mass and Forward Migration Indices (FMI) both indicate the directed cell migration. The center of mass represents the averaged point of all cell endpoints. Its x and y values indicate the direction in which the group of cells primarily traveled.

The FMI represent the efficiency of the forward migration of cells, in our case in relation to the y-axis (Equation 3). The larger the index on an axis, the stronger is the chemotactic effect on this axis. For simplification, it is assumed that the y-axis is parallel to the direction of the chemotactic gradient.

$$FMI = (distance\ on\ y-axis)/(accumulated\ distance)$$

**Equation 3 The forward migration index (FMI) indicates the efficiency of forward migration**

The value of directionality is calculated by comparing Euclidean and accumulated distance (See figure 16). It indicates the directness of the cell trajectories. A directionality of 1 equals a straight-line migration from start to endpoint.

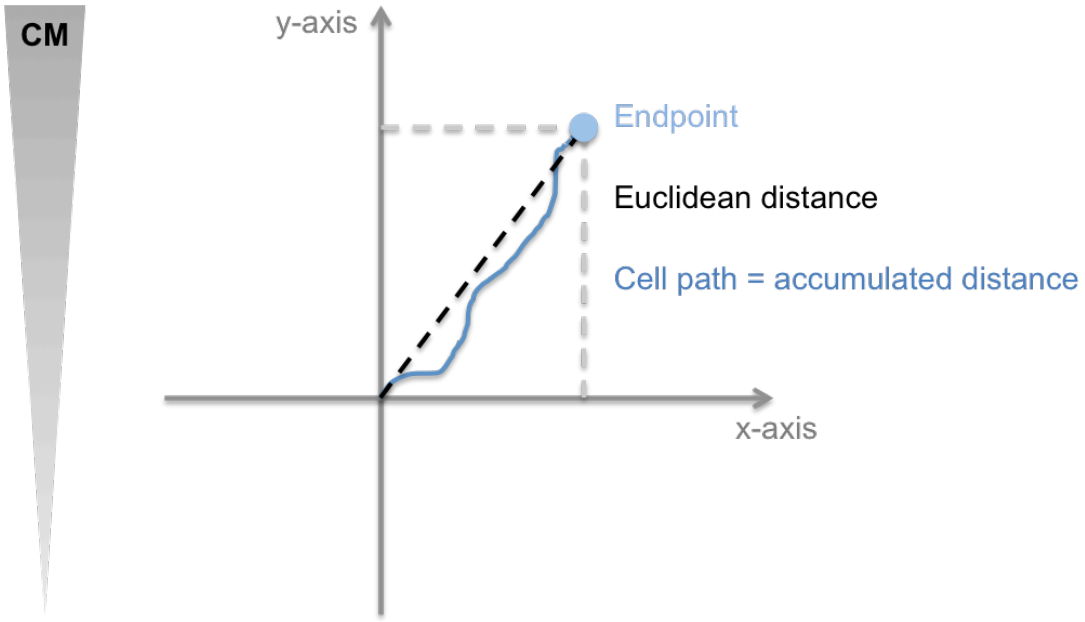



**Figure 16 Nomenclature of distances in migration experiments**

*An exemplary track of a cell is shown (cell path = accumulated distance; blue). It is assumed that the y-axis is parallel to the direction of the chemotactic gradient (CM: Gradient of tumor conditioned medium). Euclidean distance indicates the most direct connection between starting and endpoint. In comparison to the accumulated distance which indicates the total distance a cell has traveled. The parameter directionality compares Euclidean with accumulated distance.*

***Statistical analysis***
Migration data was analyzed with Prism software using the Mann-Whitney-test, an unpaired test for non-parametric data.

***Gaussia* Luciferase Assay**
The principle of the luciferase reporter makes use of a secreted version of the marine copepod *Gaussia* princeps' protein; this luciferase catalyzes the oxidation of the substrate coelenterazine in a reaction that produces light. *Gaussia* activity is assayed from supernatant of cells which have been transfected with *Gaussia* Luciferase expressing plasmids using a luminometer. The luminescence measured is proportional to the amount of enzyme produced, which reflects the level of transcription. In reporter plasmids the activation of a target gene was driving the production of the *Gaussia* Luciferase.

A BioLux *Gaussia* Luciferase Flex Assay Kit from New England BioLabs was used according to the manufacturer's manual. Per sample, 0.5µl substrate, 8µl stabilizer and 50µl assay puffer were mixed and incubated at room temperature protected from light for 25min. In 5ml tubes 50µl of the mix and 20µl of the cells' supernatant were incubated together for a minimum of 45sec. Measurements on the photometer were conducted with an integration time of 10.0 sec.

**Coculture experiments**
Once reporter cells were adherent, tumor cells or MM6 cells were added. If not indicated differently, $10^4$ reporter cells were seeded in a 96 well the day before adding cells for coculture. A 96 well contained cells in 100µl medium with 5% FCS. *Gaussia* Luciferase was measured 48h after tumor cells were added.
Using microscopy, cell viability was confirmed before measuring *Gaussia* Luciferase. In order to precisely differentiate living from dead cells, 10x Höchst-PI was added to the supernatant 1:10 and viability was checked with fluorescent microscopy.

**Supernatant experiments**
Supernatant was obtained from 100% confluent tumor cells, which were washed with PBS and cultured in DMEM without FCS for three days. Conditioned supernatant was centrifuged at 350xg for 5min to remove cells and cell debris. Aliquots were frozen and stored at -20°C.

MM6 supernatant was generated in MM6-medium without FCS (RPMI 1% PS, MEM 1x, Insulin 900µg/ml, 1mM Oxalacetic acid, 5mM Pyruvic acid). After three days cells were centrifuged and supernatant was frozen. Concentrated supernatant was obtained with Amicon centrifugal filters (10kDalton, protein concentration and desalting) and centrifugation till flow-through was seen (around 20min).
Reporter cells were seeded in a 96 well with a total of 100µl volume. The next day the medium was changed to 100µl conditioned supernatant. *Gaussia* Luciferase was measured 24h afterwards.

**Fluorescence microscopy of cells**
Fluorescence microscopy is a form of light microscopy based on the physical effect of fluorescence. Fluorochromes are activated by light of a specific wavelength and emit light of another wavelength; specific filters secure that only the emitted light is observed.
Pictures were taken as seen through the ocular and exposure time was set accordingly, gain was set to 1. Bright field and fluorescent pictures were taken of a representative spot of the cell culture plate and overlays were generated in ImageJ.
In parallel, to detect and exclude autofluorescence artifacts the probe was also examined in other filters. In order to avoid photobleaching cells were exposed to fluorescent light as short as possible. Filter Y3 (BP 610/75) was used for detecting the red fluorescent protein Tomato (Emission maximum: 581 nm).

**Fluorescence activated cell scanning (FACS) analysis**
FACS is a laser-based technology and was used for detection of fluorescent proteins in stably transfected cells. FL-protein expression was measured on a BD FACS Calibur. Debris and dead cells were excluded by 7-Aminoactinomycin D (7-AAD) staining and appropriate gating. Fluorescent protein Tomato was detected in FL2 and 7-AAD in FL3, a combination of Tomato and 7-AAD made manual compensation necessary.

**Spheroid Assay**
Tumor spheroids are heterogeneous cellular aggregates characterized by hypoxic regions and necrotic centers that are thought to better reflect complex tumor biology.

Many adherent cell lines tend to attach to each other when lacking the possibility to grow in monolayer. Tumor spheroids, 3D in vitro tumor models, were generated using the hanging-drop method. Tumor cell suspensions of $2.4*10^4$ cells/ml were seeded in drops of 25µl in a petri dish lid, flipped, put on the bottom filled with PBS and cultured in these hanging drops for three days. Spheroids with a diameter of around 300µm were harvested.

The addition of MSC to HUH7 spheroids was conducted following a standardized protocol (Rühland, 2014). In a volume of 50µl $1.5*10^4$ cells per spheroid were mixed in a 1.5ml microcentrifuge tube. After shaking for 30min at 37°C, cells that had not attached were washed away. Spheroids were then seeded back into hanging drops and incubated for 24h. Subsequently, they were fixed in 100µl 4% paraformaldehyde, washed PBS and stored at 4°C until imaging.

Finally, 3D imaging was conducted in Martinsried using light sheet microscopy.

Invasion assays, imaging and analysis were conducted in cooperation with the biological doctoral student Svenja Rühland.

## Statistical analysis

Migration assays were conducted in duplicate and repeated, yielding four times 25 tracks per condition which were analyzed applying Mann-Whitney test.

*Gaussia* Luciferase experiments were conducted at least in triplicate, repeated and the analysis of a representative experiment is shown. Graphs indicate mean ± standard deviation. Data was analyzed using student's t-test for Gaussian distributed data.

Statistical significance was marked in the graphs with p-values < 0.05 (*), < 0.01 (**) and < 0.001 (***) as determined by the software Prism (GraphPad Software, La Jolla).

# Results

## Directed migration of MSCs towards tumor-derived signals

To optimize the therapeutic application of MSCs, it is important to better understand their migratory behavior. Tumor tropsim has been demonstrated in vitro and in vivo for MSCs, however the general efficiency can vary between preparations, and some MSCs also migrate to other organs. This can limit therapeutic potential of modified MSCs as healthy tissue can also be damaged by application of the therapeutic agent. Preactivation of MSCs with pathway modulators, so-called MSC priming, is a relatively easy and fast way of potentially influencing MSC migration and tropism.
Wnt and Hedgehog (HH) signaling crosstalk at a molecular level and can be seen as part of a larger regulatory network controlling fundamental aspects of cell biology (Angers & Moon, 2009). Those pathways play key roles in development and alterations can lead to cancer. We then set out to determine if these pathways can influence directed MSC migration.

HUH7 tumor conditioned medium is a chemoattractant for MSCs (Endaya et al., 2017; Schmohl et al., 2015), this was confirmed by the observation that the center of mass of all cell trajectories was shifted towards higher concentrated tumor conditioned medium after 18h of migration, which is indicated by the orange dot on Figure 17. Moreover, the Rayleigh p-values of the medium control groups under 0.05 indicate that the cells were inhomogeneously distributed, also suggesting distribution according to a chemogradient (Figure 18f and 19f).

After prestimulation of primary MSCs with the canonical Wnt activator Wnt3a, the directed migration towards tumor conditioned medium was measured. Either 75ng/ml or 400ng/ml Wnt3a was added to MSCs in serum free medium for 24h or 48h. Then, an in vitro migration assay was conducted using ibidi µ-slides. Cell migration was followed in a 3D collagen I gel in response to a gradient of conditioned HUH7 media for 18h.
Center of mass or the Forward Migration Index (FMI) both indicate directed cell migration. The yFMI represents the efficiency of the forward migration of the cells in relation to the y-axis. The larger the index on an axis, the stronger the chemotactic

effect along this axis. For simplification, it is assumed that the y-axis is parallel to the direction of the chemotactic gradient (Figure 16). The yFMI was found to be significantly larger after prestimulation for 24h with 75ng/ml Wnt3a ($p=0.255$), and for 48h with 75ng/ml ($p<0.001$) and 400ng/ml ($p=0.0022$). Wnt3a as compared to the unstimulated control. The results show that Wnt3a prestimulation can significantly enhance MSC chemotaxis towards tumor conditioned medium (Figure 17).

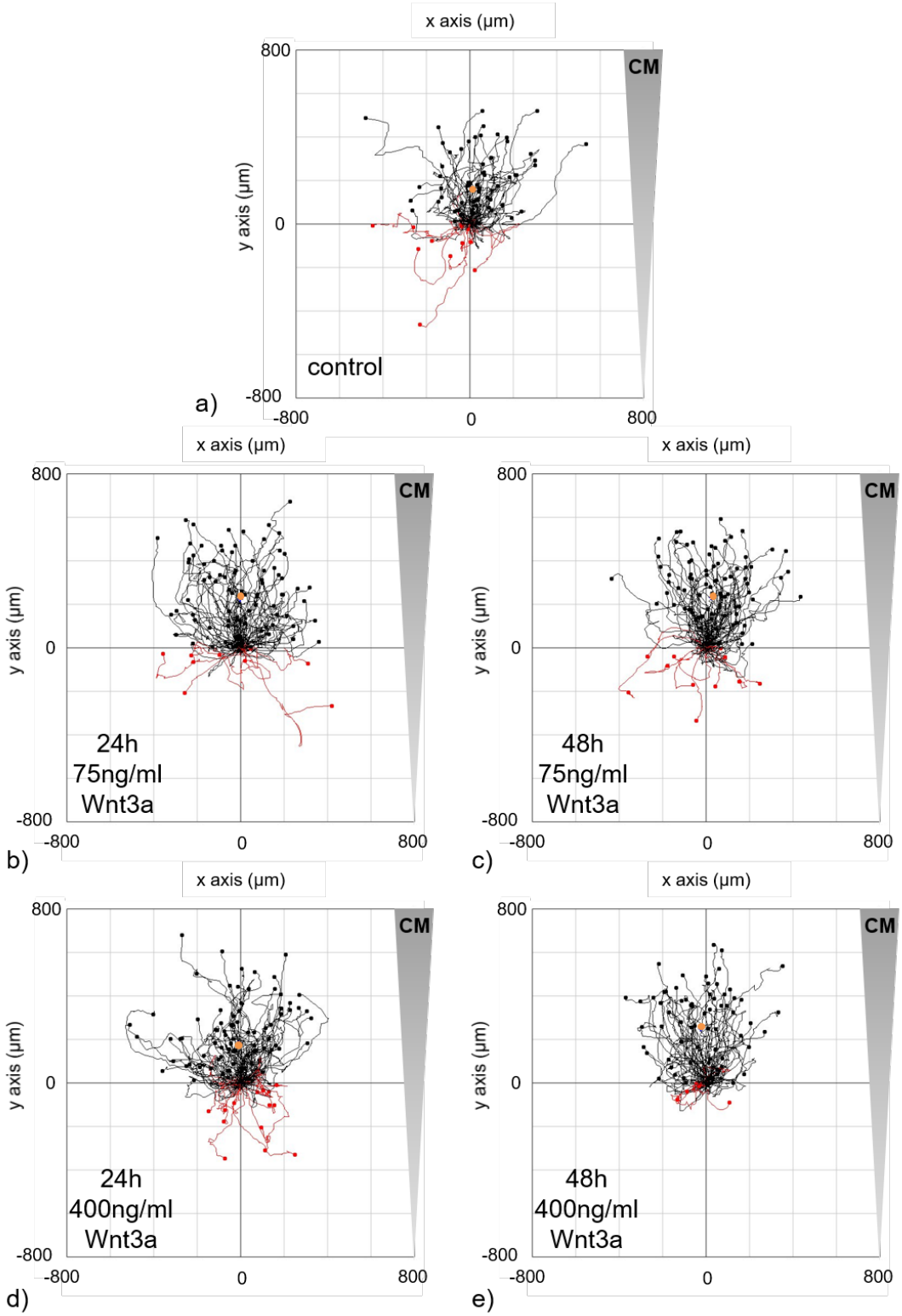



**Figure 17 Visualization of cumulative data from short-term migration experiments**

*Each track on the graph represents a path of an individual cell, the endpoint being accentuated. Black tracks follow the gradient of HUH7 conditioned medium (CM) to higher concentrations, red tracks indicate cell migration towards lower concentrations. The center of mass is marked with an orange dot. Cells were tracked for 18h after being exposed to a) control medium, 75ng/ml Wnt3a conditioned medium for b) 24h or c) 48h and 400ng/ml Wnt3a conditioned medium for d) 24h or e) 48h.*

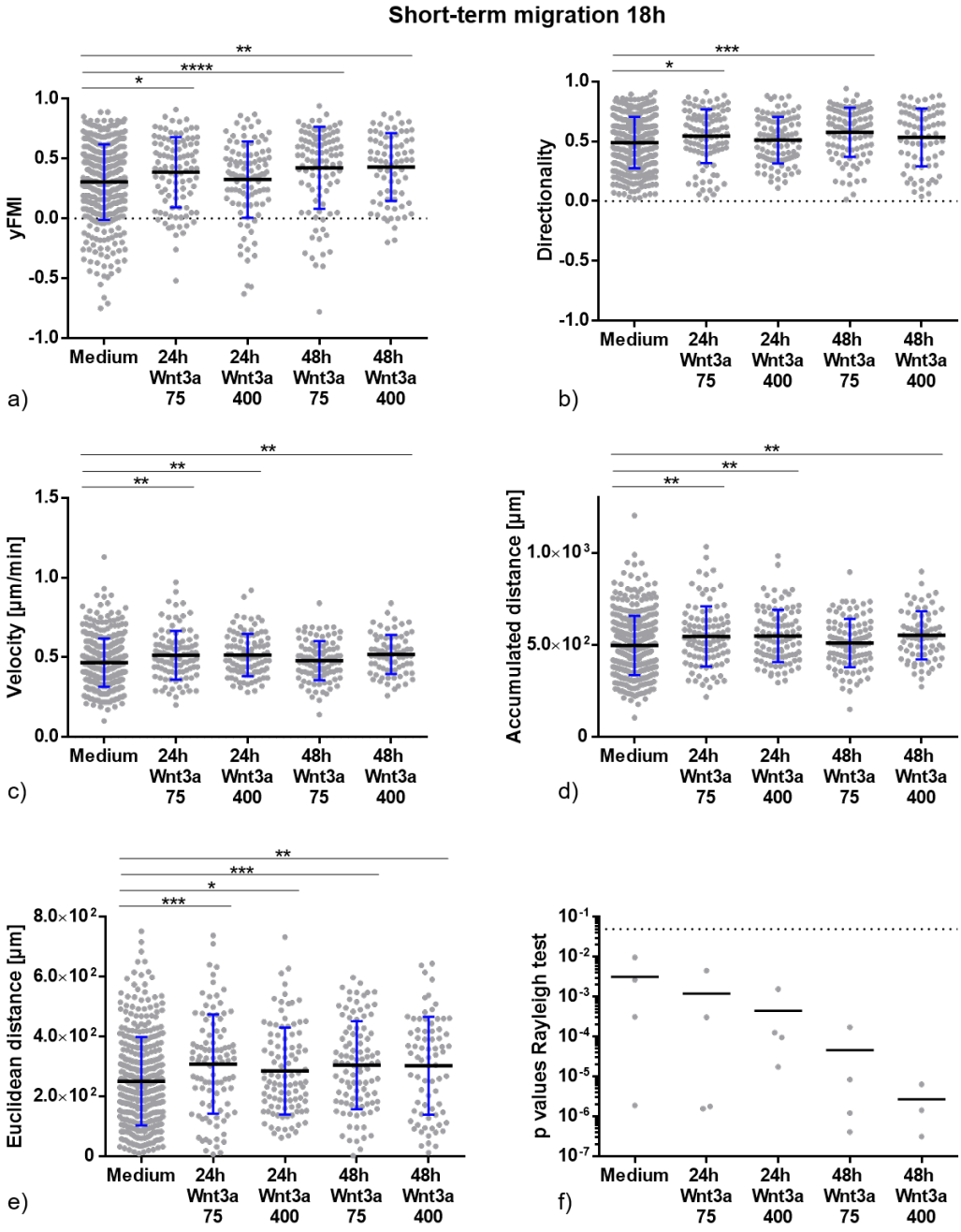



**Figure 18 Statistical analysis of cumulative short-term migration data**

*Migration parameters from MSCs prestimulated with either 75ng/ml Wnt or 400ng/ml Wnt3a, abbreviated as 75 and 400 on the graphs, for 24h and 48h are shown. Medium control graph a-e) shows results from the control group of various experiments which are not shown.*

*a) Statistical analysis of the forward migration index on y-axis (yFMI) shows significant differences between pretreated group and control which suggests that Wnt3a prestimulation enhances MSCs chemotaxis towards tumor conditioned medium. b) Directionality is calculated by dividing Euclidean by accumulated distance. Wnt3a prestimulation for 24h or 48h with 75ng/ml enhances directionality significantly which means cell trajectories become more direct from start- to endpoint. c)-e) Wnt3a prestimulation influences velocity, accumulated and Euclidean distance. f) Rayleigh p-values indicate how homogeneous the distribution of cells is; p-values under 0.05 suggest an inhomogeneous distribution, for example because cells followed the chemotactic gradient and distribution is not random anymore. (Mean ± standard deviation, Mann-Whitney-test)*

In the short-term migration studies, an enhanced directed MSC migration was observed after Wnt3a prestimulation. To investigate whether these changes of migratory behavior were permanent or transient a long-term migration experiment was then conducted.

After stimulation with 75ng/ml or 400ng/ml Wnt3a for 24h or 48h cells were cultured for two to three more cell passages, and a new migration assay was performed. No memory effect was observed, meaning enhanced chemotactic parameters FMI and directionality, were no longer enhanced by prestimulation in long-term (a week or more after stimulation), in contrast to what was seen in short-term (24-48 hrs) (Figure 19a-b and 18a-b).

Thus, the influence of Wnt3a priming on MSCs on directed migration appears to be transient and reversible.

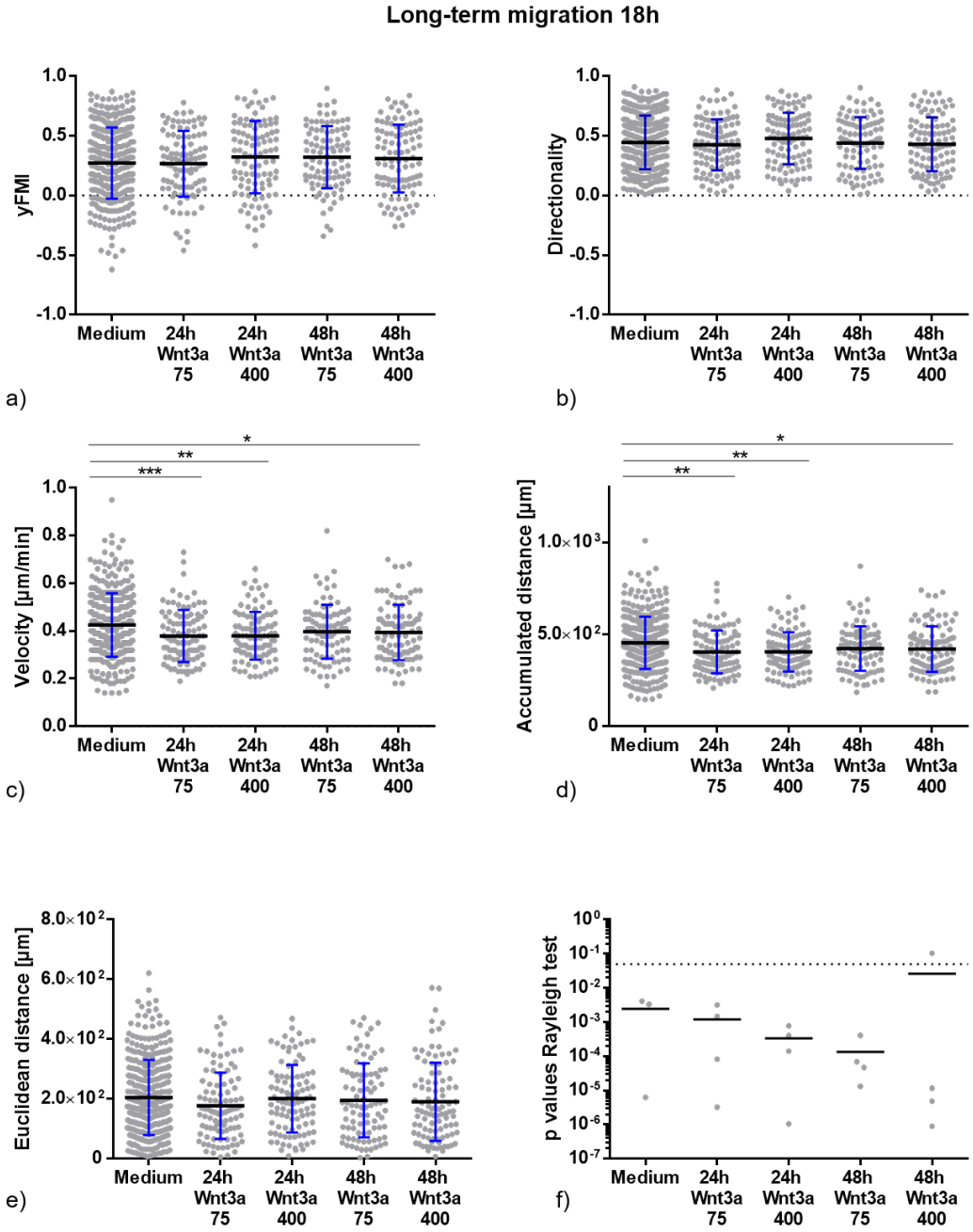



**Figure 19 Statistical analysis of cumulative long-term migration data**

*Migration parameters obtained from a migration assay with MSCs two to three passages (a week or more) after being stimulated with either 75ng/ml Wnt or 400ng/ml Wnt3a, which is abbreviated on the graph with 75 and 400 respectively, for 24h or 48h are shown. Medium control graph a-e) shows results from the control group of various experiments which are not shown.*

*a) The enhanced migratory potential of Wnt3a pretreated MSCs is temporary and reversible. Statistical analysis of the forward migration index (FMI) shows no significant differences between pretreated group and control which suggests the chemotactic enhancing effects from Wnt3a prestimulation which is observed in the short-term migration, is not detectable anymore. b) Directionality is calculated by dividing Euclidean by accumulated distance. Directionality of cell paths is not different between pretreated MSCs and control. c)-e) Wnt3a prestimulation influences velocity, accumulated but not Euclidean distance. f) Rayleigh p-values indicate how homogeneous the distribution of cells is; p-values under 0.05 suggest an inhomogeneous distribution, for example because cells followed the chemotactic gradient and distribution is not random anymore. (Mean ± standard deviation, Mann-Whitney-test)*

## Reporter cells

Canonical Wnt and HH signaling are highly conserved pathways involved in carcinogenesis and differentiation. The ligands activating those pathways can be found in tumor environments and can condition cells within the tumor. We then sought to determine if the Wnt or HH response of MSC to tumor derived ligands could lead to activation. The central question was to determine if the synthetic promoters are candidates for refined gene-directed enzyme-based MSC therapy.

### Canonical Wnt- and Hedgehog-responsive reporter iMSCs

As a first step, Sleeping Beauty transposon-based reporter plasmids with the respective synthetic promoter were generated, and stably transfected into iMSC. *Gaussia* Luciferase secretion into the growth medium was used as a read-out for pathway activation/reporter promoter activity.

The functionality of the reporter constructs was verified by stable cotransfection of an expression construct with a reporter plasmid in iMSCs. The doxycycline inducible expression constructs TCF7 (Expression vector CMV TO TCF) and Gli1 (Expression vector CMV TO Gli1) were used to activate canonical Wnt and HH reporter cells respectively. Various doxycycline concentrations were tested to identify the optimal stimulation levels which were found between 0.1 and 1µg/ml doxycycline. At higher

concentrations, beginning at 5µg/ml, toxic effects on cells were observed. Stimulation with 1µg/ml doxycycline for 72h of iMSCs stably transfected with Bar-TCF7 showed 12.3 fold increase relative to the unstimulated control, whereas HH-Gli cells showed a 2.9 fold level of induction (Figure 20).

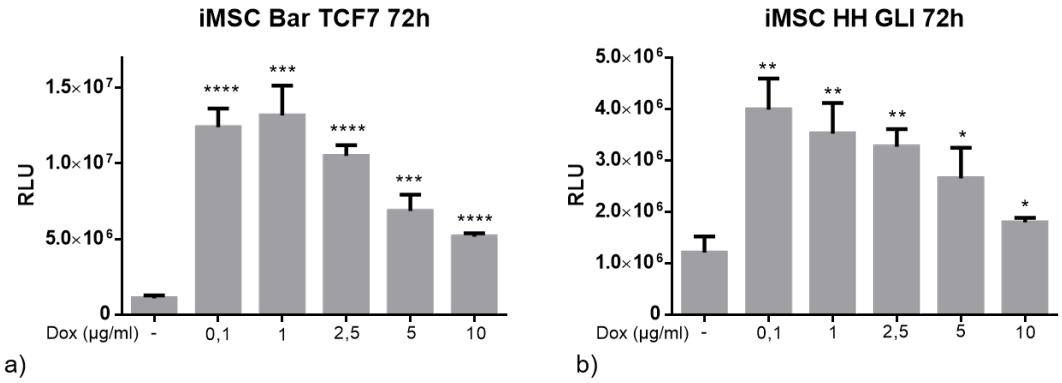



**Figure 20 Test of doxycycline inducible overexpression constructs stably transfected in iMSC**

*Stably engineered iMSC established using a reporter plasmid cotransfected with respective doxycycline (dox) inducible transcription factor construct. Various dox concentrations were then tested, 1µg/ml yielding highest fold-change to unstimulated control and was therefore used in subsequent experiments. Gaussia Luciferase was measured 72h after stimulation. a) canonical Wnt reporter (Bar) with TCF7 overexpression. b) Hedgehog reporter (HH) with GLI overexpression. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

Stably engineered iMSCs containing either the Bar or control plasmids were stimulated with different concentrations of recombinant human Wnt3a or lithium chloride (LiCl). Lithium chloride`s toxic effects on cells was observed at 50mM, being more severe in cells stimulated without FCS than with FCS present (Data not sown). Stimulation with Wnt3a and LiCl was observed in Wnt-responsive reporter cells and no stimulation was found in cells stably transfected with the control plasmid. Hereby, unspecific activation of the reporter cells, for example through upregulated metabolism, was excluded (Figure 21a).

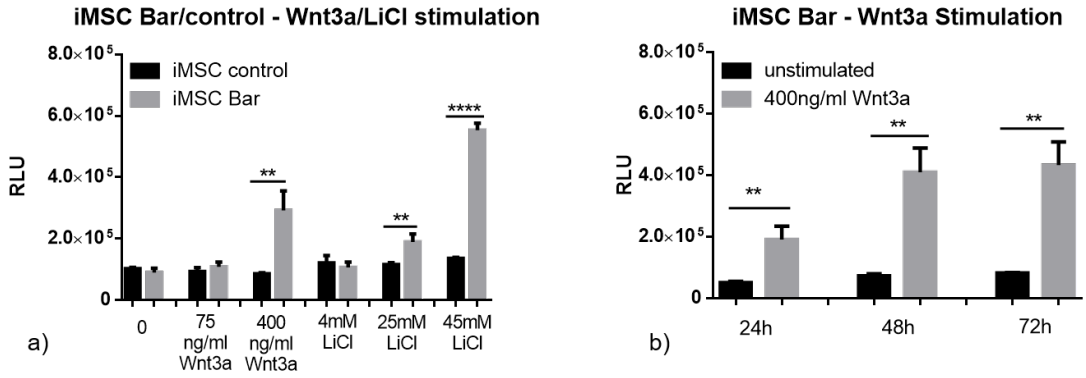



**Figure 21 Stably transfected Bar iMSCs can be stimulated with Wnt3a or lithium chloride**

*a) In contrast to iMSC stably transfected with a control plasmid, iMSC Bar reporter can be stimulated with Wnt3a or lithium chloride (LiCl), thereby excluding unspecific stimulation. b) Gaussia Luciferase measurement at 24h, 48h and 72h after stimulation with 400ng/ml Wnt3a, showed the highest stimulation at 48h with 5.6 fold-change to the unstimulated control. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

Stably transfected iMSCs were stimulated with 400ng/ml recombinant human Wnt3a for 24h, 72h or 96h. Gaussia Luciferase measurement after 48h showed the highest fold change to the unstimulated control with 5.6 fold, compared to 3.7 and 5.2 fold at 24h and 96h respectively (Figure 21b).

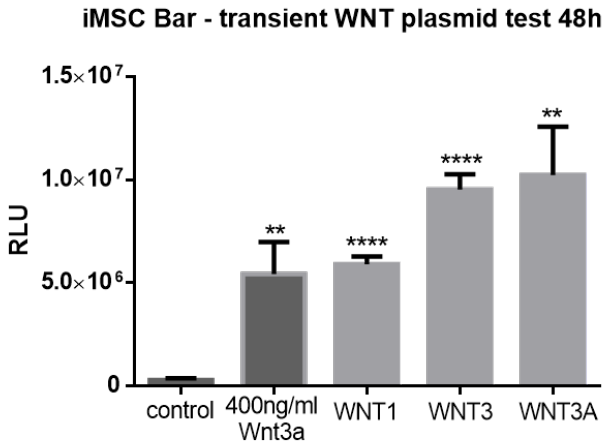



**Figure 22 Functionality of the Wnt protein producing plasmids WNT1, WNT3 and WNT3A is shown in Wnt reporter iMSC**

*Transiently transfected with WNT1, WNT3 or WNT3A plasmid iMSC Bar are activated compared to mock transfected control. Gaussia Luciferase was measured 48h after the transient transfection with lipofectmine. As an additional control mock transfected iMSC Bar were stimulated with the recombinant protein Wnt3a. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

Constitutively WNT1, WNT3 or WNT3A gene expressing plasmids were cloned to produce control Wnt proteins. After transient transfection into iMSC Bar reporter cells, all constructs lead to significantly increased *Gaussia* Luciferase signal, verifying their functionality (Figure 22). The proteins Wnt1, Wnt3 and Wnt3a are known as classical activators of the canonical Wnt signaling pathway. In addition, expression constructs transiently transfected into iMSC showed enhanced Wnt signaling. Suggesting that, these plasmids can also be used as positive controls for Wnt reporter cells.

**Canonical Wnt and Hedgehog-responsive reporter primary MSCs**

The functionality and suitability of the reporter plasmids for stable transfection in immortalized cell lines and primary MSCs was verified.

Primary MSCs were stably transfected using Neon electroporation. Induction of the expression plasmid TCF7 with 1µg/ml doxycycline lead to increased Bar reporter activity and confirmed the functionality of the reporter. Moreover, the presence of the canonical Wnt signaling cascade was verified using recombinant human Wnt3a added to the supernatant. Combination of doxycycline derepression and Wnt3a stimulation showed additive effects. The optimal time point for *Gaussia* luciferase measurement after doxycycline stimulation was found at 72h, drawn from fold-change to control (Figure 23).

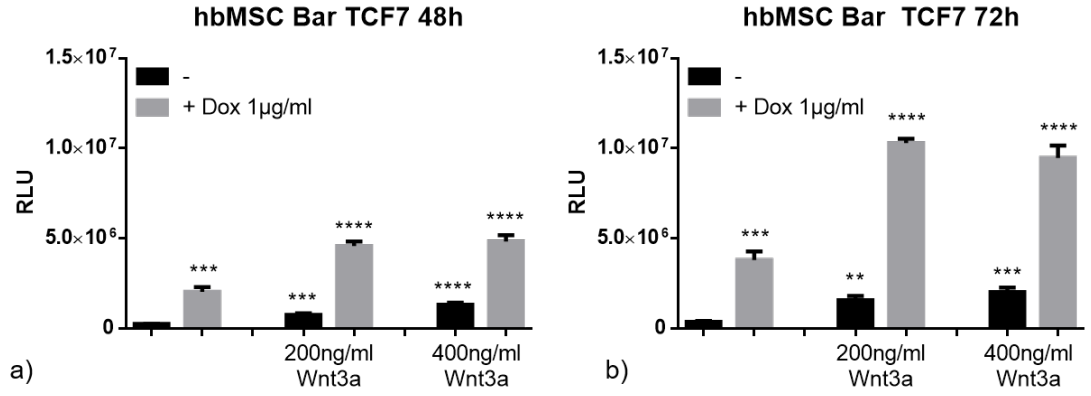



**Figure 23 Primary MSCs can be stably cotransfected with the Wnt reporter (pcDNA-Gluc3-CMVMin-Bar) and inducible expression plasmid (Expression vector CMV-TO TCF7)**

*Functionality of the reporter cells, the inducible expression construct TCF7 and the canonical Wnt signaling cascade in stably transfected primary human bone marrow derived MSCs (hbMSC) was verified. Stably cotransfected hbMSCs with Wnt signaling reporter (Bar) and doxycycline inducible TCF7 expression construct showed increased reporter activity upon stimulation with 1µg/ml doxycycline (dox). Stimulation with recombinant human Wnt3a (Wnt3a) was possible, verifying the presence of the canonical Wnt signaling cascade in hbMSCs. Gaussia Luciferase was measured a) 48h and b) 72h after stimulation. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

Primary hbMSC HH reporter cells stably transfected with the doxycycline inducible expression construct Gli1, showed increased *Gaussia* Luciferase signal upon stimulation with 1µg/ml doxycycline. The ideal time point for *Gaussia* Luciferase measurement after doxycycline stimulation was found at 72h with 1.8-fold induction

compared to unstimulated control. Thereby constructs' functionality in hbMSC was shown (Figure 24).

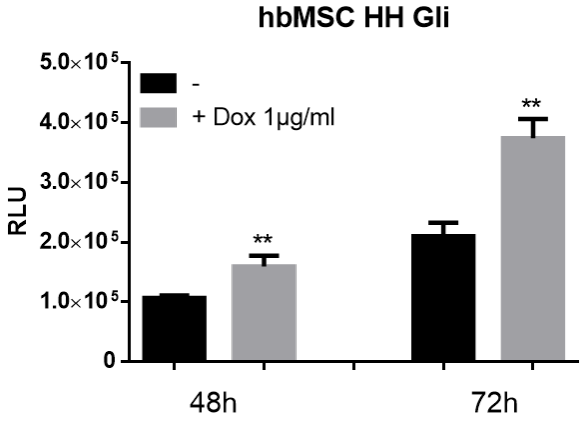



**Figure 24 Primary MSCs can be stably transfected with the Hedgehog reporter (pcDNA-Gluc3-CMVMin-HH) and inducible expression plasmid Gli1 (Expression vector CMV-TO Gli1)**

*Functionality of the Hedgehog (HH) reporter and the inducible Gli1 expression plasmid were verified in primary hbMSC. Stably transfected hbMSC HH reporter cells with doxycycline inducible Gli1 expression constructs show induction upon stimulation with 1µg/ml doxycycline (dox). Fold-changes compared to unstimulated control constitute 1.5 fold after 48h and 1.8 fold after 72h. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

**Coculture experiments**

In the next set of experiments, we sought to determine if the Wnt or HH response in MSC could be activated by contact with cells representing leukocytes within the tumor milieu such as macrophages, or by tumor cells.

The influence of tumor coculture on reporter plasmids stably transfected into Hek293 was tested, because those cells were easy to transfect as well as to culture and therefore, were used in proof of principle experiments. Secondly, iMSC reporter cells were cocultured with tumor cell lines to investigate the tumor milieu`s influence on those reporter cells.

Bar and HH reporter plasmids stably transfected into Hek293 were stimulated in coculture and with supernatant of SW480, HT29, HUH7 and HT1080 tumor cell line and MM6, a macrophage cell line (Refer to page 25-27 for more information about the cell lines). Significantly enhanced reporter activity was observed in Hek Bar and Hek HH reporter cells. In contrast, stably transfected iMSC Bar or iMSC HH did not show increased reporter activity in either coculture (Figure 25).

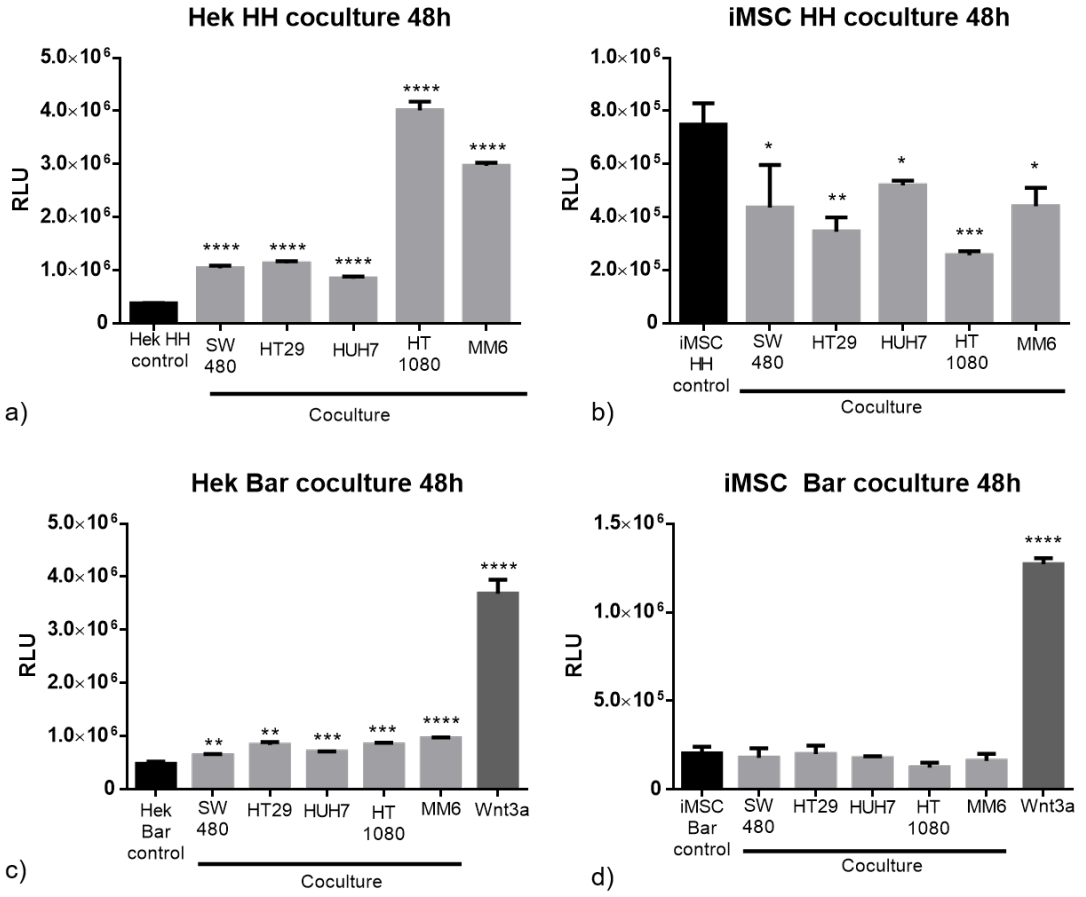



**Figure 25 Coculture of the cell lines Hek293 or iMSC with HH or Wnt reporter with tumor and macrophage cell lines**

*Hek293 (Hek) cells stably transfected with either Wnt (Bar) or Hedgehog (HH) reporter can be stimulated in coculture with SW480, HT29, HUH7 and HT1080 tumor cell lines and the MM6 macrophage cell line. As positive control in Bar reporter cells 400ng/ml Wnt3a was used. b) iMSC HH coculture showed lower Gaussia Luciferase signal, whereas d) iMSC Bar showed no significant difference from control. In the experimental set-up, $10^4$ reporter cells were added the day before $4*10^4$ tumor or macrophage cells were added to a 96 well. Gaussia Luciferase was measured after 48h of coculture. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

In Hek HH an increasing reporter activity is found with increasing MM6 coculture cell numbers (Figure 26). Two different iMSC cell lines were tested to emphasize the finding and to exclude specific problems with one immortalized cell line. Neither in

peripheral blood derived nor in bone marrow derived stably transfected iMSC HH stimulation could be observed (Figure 26).

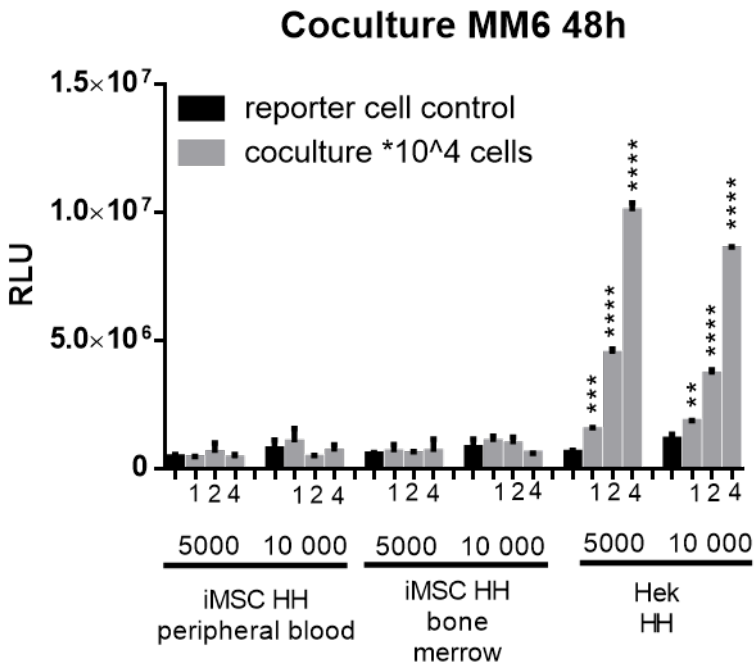



**Figure 26 Hek293 and two different iMSC lines in coculture with different MM6 cell numbers**

*Black columns represent reporter cells only as control, either 5 000 or 10 000 were seeded the day before MM6 cells were added. Grey columns represent coculture of the respective HH reporter cell line with 1*10$^4$, 2*10$^4$ or 4*10$^4$ MM6 cells. Dose dependent stimulation was observed in stably transfected Hek HH cells, but not in stably transfected iMSC HH, neither peripheral blood derived cells nor bone marrow derived cells. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

As iMSCs are bigger in size than Hek293 cells, also lower cell numbers were tested to exclude that competition between reporter and MM6 cells obscures mild effects. However, also when $10^3$ reporter cells were seeded the day before coculture with 2*$10^3$, 4*$10^3$ or 6*$10^3$ MM6 or HT1080 cells, no stimulation was observed in reporter iMSC (Figure 27). Because HT1080 and MM6 cells showed higher stimulation of

Hek HH reporter activity than other cells tested in coculture, those two cell lines were used.

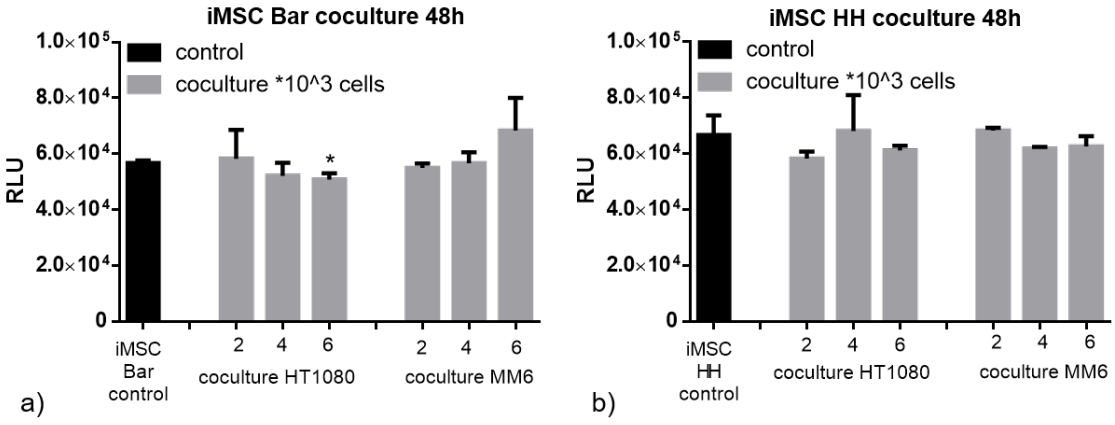



**Figure 27 No stimulation of iMSC Wnt or Hedgehog reporter cells in coculture with low cell number of HT1080 or MM6 cells can be observed**

*Coculture with lower cell numbers was conducted in order to exclude competition for space was obscuring stimulatory effects. In the experimental set-up, $10^3$ reporter cells were seeded the day before $2*10^3$, $4*10^3$ or $6*10^3$ HT1080 or MM6 cells were added to a 96 well. Gaussia Luciferase was measured after 48h. No stimulation neither of iMSC Bar nor iMSC HH was observed. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

**Supernatant experiments**

We next sought to determine if soluble factors secreted by tumor cells or macrophages lead to activation of canonical Wnt or HH response in MSC.

The reporter cells were then exposed to supernatants of various tumor cell lines and a macrophage cell line to investigate whether soluble factors were mediators of the stimulation observed in coculture experiments. In contrast to coculture experiments, supernatant experiments mimic tumor milieu without competition for space and regardless of differences in proliferation rates among cell lines, and therefore, can be better controlled.

Reporter cells were stimulated with tumor cell line supernatant of SW480, HT29, HUH7 and HT1080. Hek HH and Hek Bar cells showed significant increase in reporter activity (Figure 28a, c). In contrast to iMSC reporter cells, which did not show a significant difference from control (Figure 28b, d). Human epidermal growth factor (EGF), in a concentration of 25ng/ml was able to stimulate Hek HH reporter cells, but not iMSC HH (Figure 28a, b).

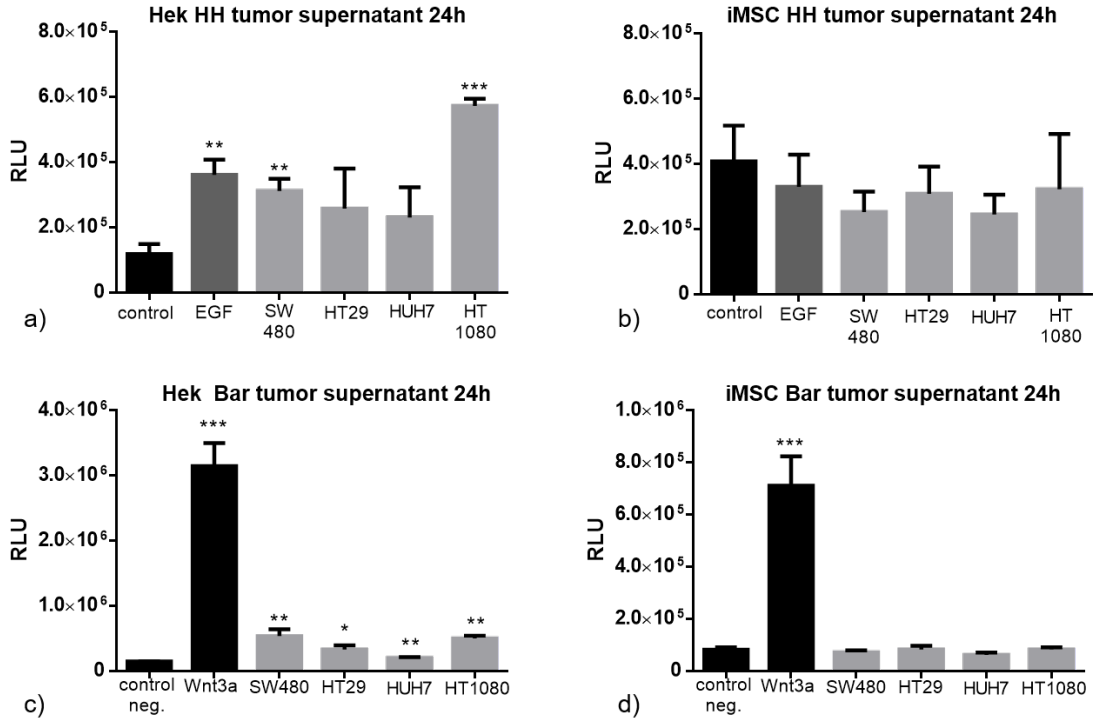



**Figure 28 Various tumor cell supernatants (SW480, HT29, HUH7 or HT1080) added to the cell lines Hek293 or iMSC with HH or Wnt reporter**

*Hek293 (Hek) and iMSC reporter cells were cultured in tumor cell line conditioned serum free DMEM medium for 24h, before measuring Gaussia Luciferase. a) Hek Hedegehog reporter (HH), b) iMSC HH, c) Hek Wnt reporter (Bar) and d) iMSC Bar. Significant increase in reporter activity was observed in Hek reporter cells, but not in iMSCs. As a positive control for the Bar reporter stimulation with 400ng/ml Wnt3a was used. Human epidermal growth factor (EGF), in a concentration of 25ng/ml was able to stimulate Hek HH reporter cells, but not iMSC HH. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

Supernatant of the macrophage cell line MM6 was able to stimulate Hek HH reporter cells and supernatant 8-fold concentrated via Amicon (10kDalton centrifugal filters) increased the effect (Figure 29a). Hek Bar cells only showed significantly enhanced reporter activity with concentrated supernatant (Figure 29c). In contrast, iMSC HH or Bar reporter showed decreased reporter activity in presence of concentrated supernatant (Figure 29b, d).

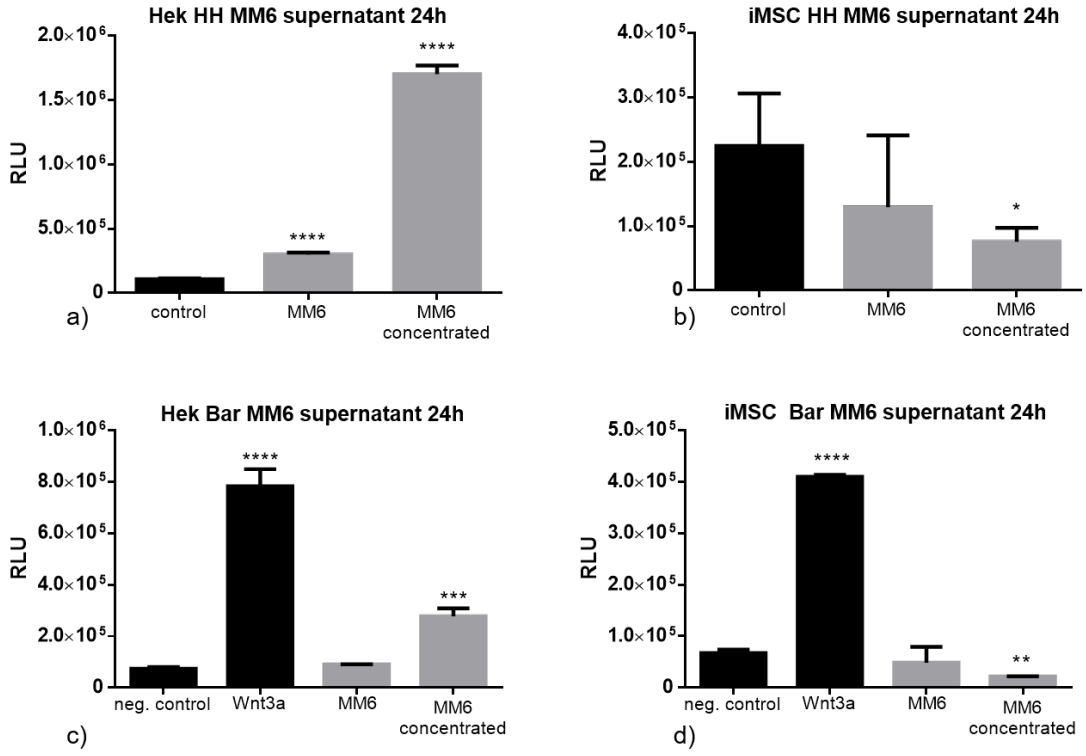



**Figure 29 MM6 macrophage supernatant added to the cell lines Hek293 or iMSC with HH or Wnt reporter**

*a) Hek Hedegehog reporter (HH), b) iMSC HH, c) Hek Wnt reporter (Bar) and d) iMSC Bar were cultured in MM6 conditioned serum free MM6 medium for 24h, before measuring Gaussia Luciferase. Whereas MM6 and 8-fold concentrated MM6 conditioned medium stimulated Hek HH, and concentrated medium Hek Bar cells, iMSC reporter cells even showed decreased reporter activity. As a positive control for Bar reporter cells 400ng/ml Wnt3a was used. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

**Coculture and supernatant experiments in primary cells**

In order to investigate whether the observed effects in iMSC were due to issues related to an immortalized MSC cell line or generally applicable to primary MSCs, select coculture and supernatant experiments were repeated with primary human bone marrow derived MSC (hbMSC). Unfortunately, stably transfected reporter hbMSC were difficult to generate and culture and only a view experiments could be conducted with the limited cell numbers. Primary Bar reporter cells could not be activated in coculture nor with supernatant of HT1080 cells. Viability of cells was checked through light and fluorescence microscopy after adding Höchst-PI (Figure 30c). Höchst is a living cell dye highlighting nuclei of eucaryotic cells and PI is a dead cell stain, penetrating membranes of dying or dead cells.

HH reporter cells cocultured with HT1080 showed decreased reporter activity compared to unstimulated control (Figure 31).

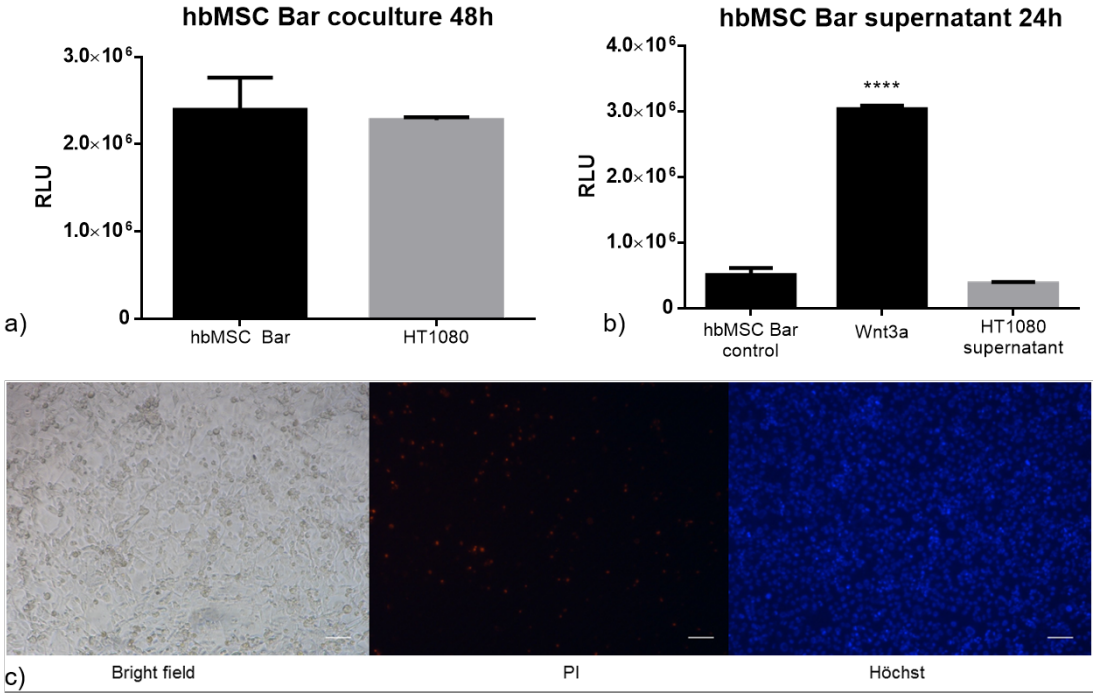



**Figure 30 Primary Wnt responsive (Bar) MSCs cannot be stimulated in coculture with HT1080 cells nor with its supernatant**

*Primary human bone marrow derived mesenchymal stem cells (hbMSC) Wnt reporter (Bar) cells could not be activated in a) coculture with 4*10$^4$ HT1080 cells nor with b) HT1080 supernatant. Positive control with 400ng/ml Wnt3a shows*

*significantly enhanced reporter activity. (mean + standard deviation; student's t-test; RLU = relative luciferase unit). c) Höchst-PI was added to the coculture to differentiate living from dead cells using fluorescent microscopy. A view dead cells were detected with PI, however, Höchst showed many living cells. Scale bar 100µm.*

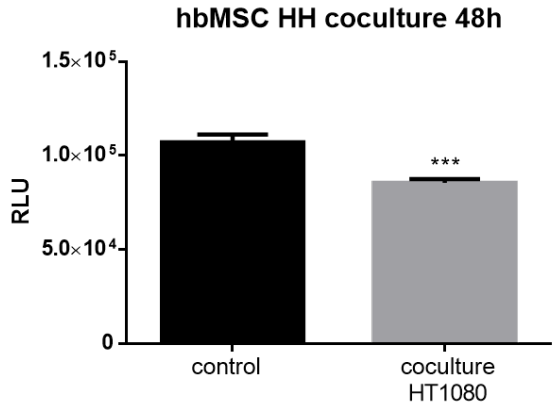



**Figure 31 Primary Hedgehog responsive (HH) MSCs in coculture with HT1080 show decreased signal**

*Primary bone marrow derived mesenchymal stem cells (hbMSC) Hedgehog (HH) reporter cells were seeded the day before 2*10$^4$ HT1080 cells were added. No stimulation was observed, instead a significantly decreased reporter signal was detected. (mean + standard deviation; student's t-test; RLU = relative luciferase unit)*

**Wnt-responsive fluorescent cells**

To investigate the tumor milieu and its potential effect on MSCs, reporter cells with a fluorescent protein read-out were established to measure promoter activity of stably transfected MSCs while invading a 3D tumor model, a so-called spheroid. Tumor spheroids are heterogeneous cellular aggregates characterized by hypoxic regions and necrotic centers that are thought to better resemble complex tumor biology.

MSC undergo invasion of the spheroid based on the activation status of the MSC, and can induce transgene expression based on the ability of the reporter MSC to respond to specific signals present in the spheroid environment.

Utilizing light sheet microscopy, fluorescence of cells, thus representing canonical Wnt signaling activity, could be measured once cells had invaded the spheroid.

Establishment of this assay should help in the characterization of tumor microenvironments in general, and whether these promoters could be useful for the induced expression of therapeutic transgenes in the context of malignancies.

Only one cell in one spheroid, out of eight spheroids in total, showed a Tomato positive iMSC, which was most likely a Tomato positive cell even unstimulated rather than representing canonical Wnt pathway activity in the tumor milieu. However, it can be concluded that Tomato fluorescence could be deteced and did not interfere with 5-Chloromethylfluorescein Diacetate (CMFDA) labelling of iMSCs (Figure 32).

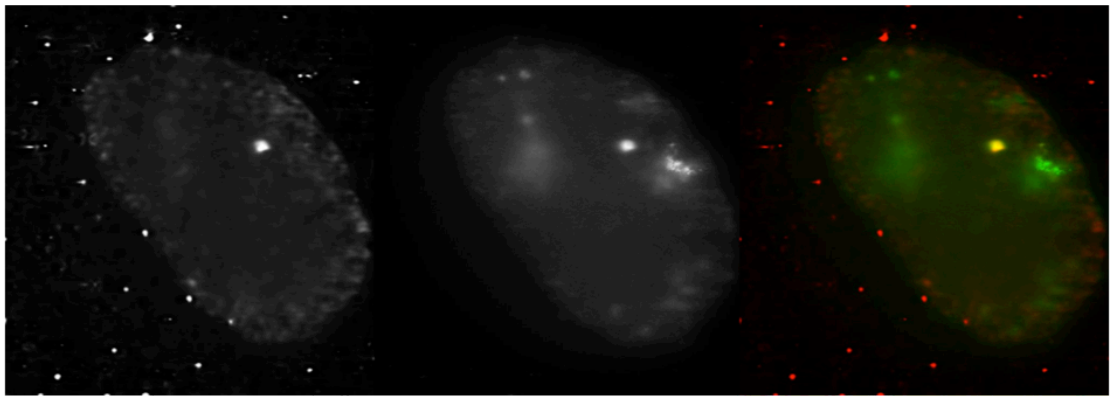



**Figure 32 Stably transfected Bar-Tomato iMSC invade a HUH7 tumor spheroid**

*Stably transfected iMSCs Bar-Tomato invade a HUH7 spheroid. Tomato fluorescence could be detected in CMFDA labelled iMSCs using lightsheet microscopy. However, the one Tomato positive cell found in one out of 8 spheroids is rather artefact than indicating Wnt signaling activity. Beats were used to calculate a 3D image from 2D images taken from five different angles (signal outside the speroid in Tomato and composite, red in the composite).*

Reporter cell function was confirmed by prestimulation with the canonical Wnt activator Wnt3a, as well as LiCl ,and measuring flourescence using FACS.
In FACS analysis, strong induction of reporter cells was observed under stimulation with 25mM LiCl, yielding 70.2% Tomato positive cells and a y-mean of 474, the more physiologic Wnt signaling activator Wnt3a showed a moderate induction of 2.6% Tomato positive cells and a y-mean of 96 (Figure 33). Dead cells were

identified using 7-AAD staining and were excluded by appropriate gating. LiCl concentrations over 45mM yielded an increased number of dead cells, especially when stimulation was performed without FCS. (Data not shown.)

Primary bone marrow derived MSCs stably transfected with the Bar-Tomato reporter plasmid show dose-dependent induction under Wnt3a stimulation in FACS analysis. Neither HUH7 nor HT1080 tumor cell supernatant show increased reporter activity (Figure 34).

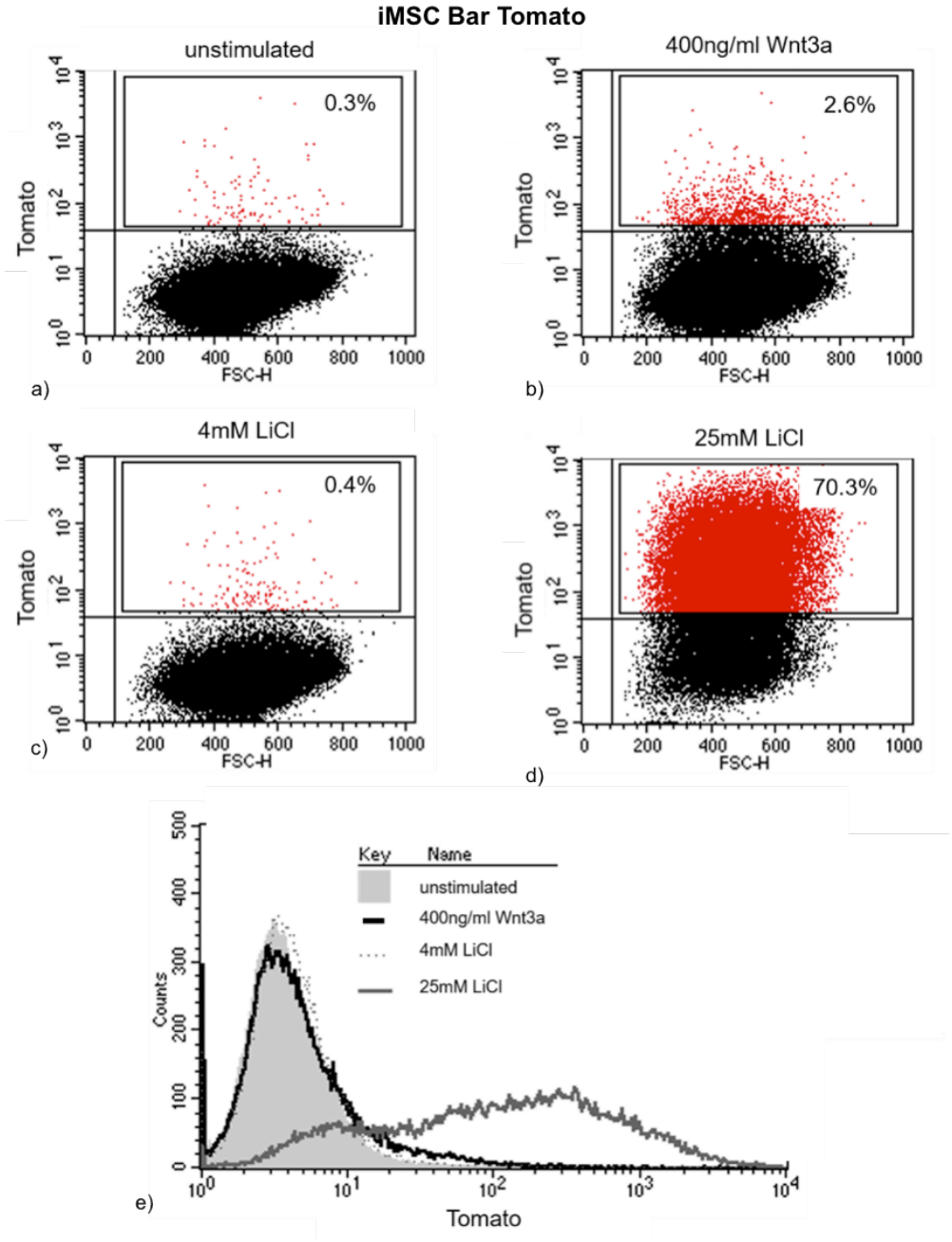



**Figure 33 FACS analysis of stably transfected Bar-Tomato iMSC under different stimulation conditions**

*FACS analysis of iMSCs stably transfected with the Bar-Tomato plasmid after stimulation with b) 400ng/ml recombinant human Wnt3a, c) 4mM or d) 25mM lithium chloride (LiCl) for 24h. e) The histogram shows the distribution of Tomato flourescence intensity its cell count. Dead cells and cell debris were excluded by 7-AAD staining and appropriate gating.*

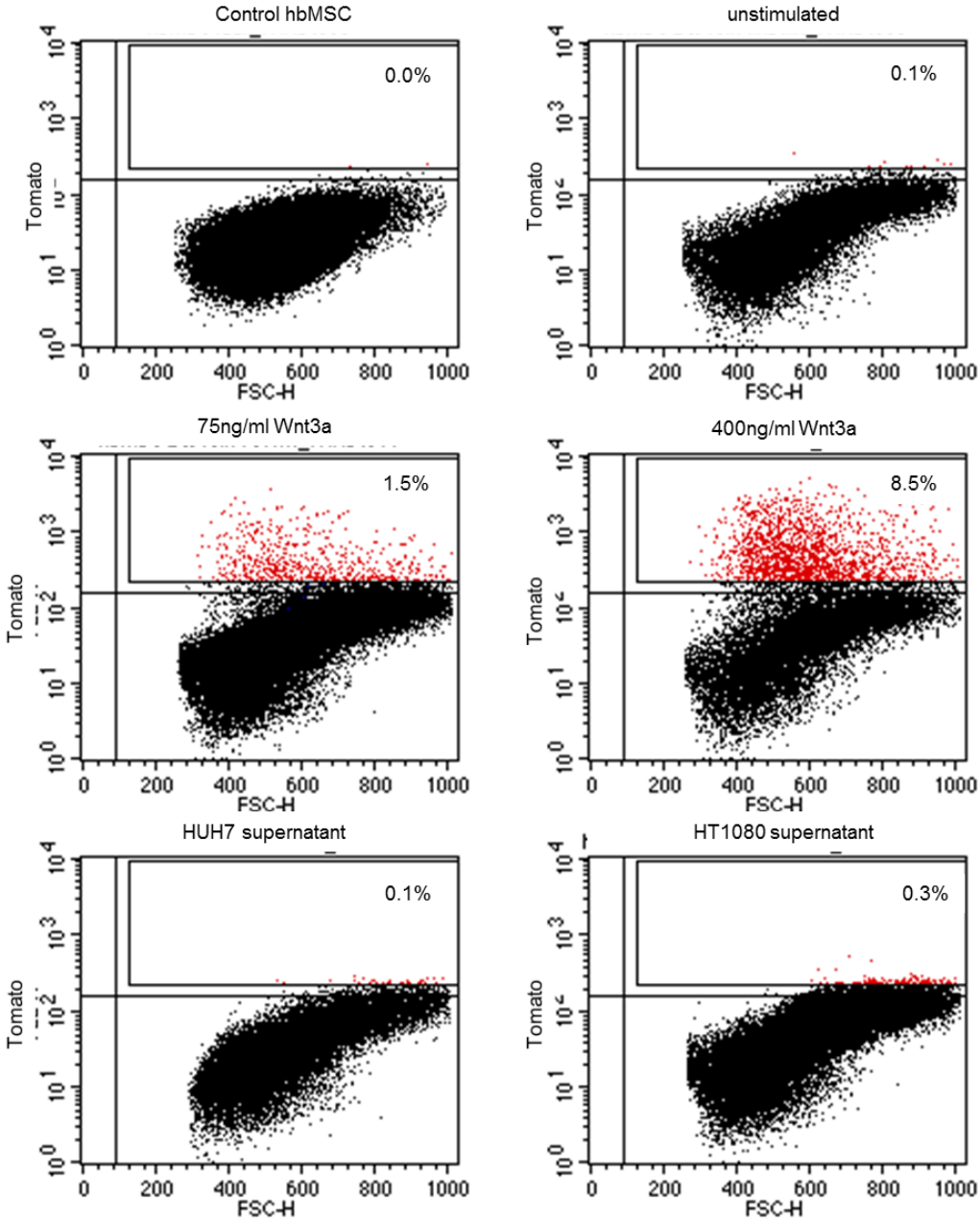



**Figure 34 FACS analysis of primary MSC stably transfected with Bar Tomato reporter under different stimulation conditions**

*FACS analysis of stably transfected primary human bone marrow derived mesenchymal stem cells (hbMSC) with a canonical Wnt reporter (Bar) and Tomato read-out. Dose dependent induction under Wnt3a stimulation was observed, however, neither HUH7 nor HT1080 supernatant stimulated reporter cells.*

# Discussion

## Influencing MSC migration and tumor tropism

MSCs are actively recruited to sites of inflammation, injury and tumor. Once MSCs reach the tumor, they can act as progenitors for cells that become part of the tumor stroma, and thus are generally thought to promote tumor progression. Genetically modified MSCs have been adapted for use as therapeutic vehicles for the delivery of therapy genes into tumor environment. Wnt and Hedgehog (HH) signaling are important pathways in development as well as differentiation and aberrant signaling is linked to carcinogenesis. Therefore, we sought to investigate their potential roles in the directed MSC migration towards tumor-derived signals.

To test this question, various migration assays were applied. These included transwell migration assays, also called modified Boyden chambers, where the transmigrated cells are quantified at a certain time point. More recently, and in comparison, µ-Slides Chemotaxis 3D now allow cell tracking through microcopy using pseudo video analysis, and therefore, can provide more detailed information at various time points. This assay is very efficient for measuring the migration of adherent cells. Parameters that can be determined in this method include the distance traveled, as well as cell distribution from which the forward migration index (FMI) and directness can be calculated. Zengel et al. proposed that the generation of a stable gradient for a longer time period, as well as tracking over long distances, is also possible with this assay (Zengel et al., 2011). For the migration experiments in this thesis, µ-Slide Chemotaxis 3D, ibdiTreat were used.

Conditioned medium (CM) generated from a hepatocellular carcinoma cell line (HUH7) was used as a tumor relevant chemoattractant. Hepatocellular carcinoma has been shown to secrete diverse factors that can influence MSC recruitment. These include; VEGF, PDGF, TGFβ, MCP-1, IL-8, TNFα, SDF-1 and HGF, as reviewed by Bayo et al. (Bayo et al., 2014).

The chemoattractant's effect on MSCs was confirmed by changes in the center of mass (Figure 17, orange dot) that shifted towards the higher concentrated "tumor" conditioned medium. Inhomogeneous cell distribution was also indicated by a Rayleigh p-value under 0.05 (Figure 18f and 19f).

MSC heterogeneity is thought to represent a potential obstacle for the application of these cells in a clinical setting. It has been proposed that even though MSCs are cultured under ISCT criteria, they remain somewhat heterogeneous. Thus, a subset of those cells may have better homing abilities in a given setting. Within individual experiments performed here, relatively high standard deviations for the FMI were observed, as some cells migrated more efficiently towards tumor CM than did others. However, repeated experiments, and an expanded number of tracked cells allowed us to identify significant differences in migratory behavior of the cell population. Four slides with 25 tracked cells each were generally analyzed per condition to identify significant differences between different parameters.

MSC experiments need to be carefully planned and well controlled, as many factors influence MSC viability as well as migration. For example, it has been suggested that increased culture confluency impairs migration (De Becker et al., 2007). Therefore, hbMSC confluency was controlled and documented in order to enhance comparability within, and between experiments.

MSC are relatively plastic cell types. They have the capacity to differentiate and are sensitive to their growth and expansion environment. It has been suggested that a pre-activation, or induced differentiation, may alter their general tropism for tumor-derived signals. In this regard, the Hedgehog and Wnt signaling pathways are strongly associated with the differentiation status of cells, as well as their ability to undergo migration. To study this in more detail, preconditioning experiments using Wnt-relevant signals were performed. Human recombinant Wnt3, a classical activator of canonical Wnt signaling, was used for preconditioning of primary human MSCs. A low and high concentration of the recombinant agent was used, namely 75ng/ml and 400ng/ml. We and others have observed in *Gaussia* Luciferase experiments that higher concentrations did not yield better stimulation of Wnt-responsive cells. The low concentration was used to avoid potential negative feedback loops due to excessive stimulation.
The distinction between chemotaxis and chemokinesis is important when interpreting migration data. Chemotaxis is defined as the directed migration of cells,

for example towards a gradient of chemokines secreted by tumor cells. By comparison, chemokinesis is defined as a general increase in the random motility of a cell. Therefore, for the accurate interpretation of migration data, various parameters need to be considered. An altered forward migration index (FMI) and directness, which both can be calculated, suggest that the stimulus exerts chemotactic effects, whereas altered velocity alone could be interpreted as enhanced chemokinetic effects. The effects of Wnt3a prestimulation on MSC migration are chemotactic, as the FMI and directness show significantly enhanced migration towards tumor CM in prestimulated MSCs. Consequently, Wnt3a prestimulation is suitable for improving MSCs' chemotaxis towards tumor-derived signals (Figure 18a-b).

Unfortunately, it was not possible to study the potential effect of Sonic Hedgehog preactivation on MSC migration. The only commercial sources of recombinant protein required extremely high concentrations of the protein to see effects, that made the experiments cost prohibitive. Attempts to generate a Shh producing plasmid and cell line failed.

Wnt signaling has been linked to the potential differentiation of cells. We then sought to determine if the changes seen with Wnt3a stimulation persisted in the treated cells. To do this, primary human MSC were again stimulated with high and low levels of the recombinant protein, and the cells were then passaged for several weeks, and then subjected to migration analysis. The general improvement of migration seen in the short term (several days) could not be demonstrated after culturing cells for two to three passages and are thus temporary and fully reversible (Figure 19).

In conclusion, Wnt3a preconditioning of MSCs influenced their directed migration towards tumor-derived signals significantly in the short-term (Figure 18). Moreover, the stimulatory effect was temporary and reversible, which on one hand, may be seen to help limit potential side effects of MSC pretreatment (Figure 19). However, further validation in vivo is necessary to evaluate the significance of these findings for potential therapeutic applications.

## Wnt and HH responsive transgenes

Canonical Wnt and HH signaling are highly conserved pathways involved in development and carcinogenesis (Takebe et al., 2015). The ligands activating those pathways can be found in tumor environments, and can in turn condition cells within the tumor environment. Investigating whether synthetic promoters designed to measure canonical Wnt and HH signaling in MSC can be activated by tumor-derived signals was used to determine if this biology was applicable for use in the tumor-induction of MSC transgenes.

### Reporter plasmids and transfection

Reporter plasmids were generated using specific synthetic promoters with secreted *Gaussia* Luciferase used as a read-out for pathway activity to efficiently measure the status of canonical Wnt and HH signaling pathways in MSC.

There are various strategies that have been developed for transducing MSCs, including virus-mediated methods (such as lentivirus, retrovirus or adenovirus) and plasmid-mediated methods (Dwyer, Khan, Barry, O'Brien, & Kerin, 2010; Zischek et al., 2009). Viruses as gene-delivery vehicles are somewhat controversial for potential human application as they show integration-site preferences that may increase chances of adverse effects, the need for extensive purification and quality control to prevent replication-competent virus, the costs associated with their production and handling, and finally, the requirement of S2 safety laboratories for viral-based engineering (Aronovich, McIvor, & Hackett, 2011). The various transfection methods described within this thesis were plasmid-mediated using either electroporation or nucleofection. A plasmid-based vector platform was specifically developed for these and related studies (Jackel et al., 2016). The plasmids were designed to include transposase-based gene transfer technology using the Sleeping Beauty (SB) transposon for stable transfections (pCMV(CAT)T7-SB100, Addgene). The SB system allowed efficient transfection and near-random integration in the host cell genome. Primary MSCs were found to be efficiently stably transfected using our vector platform and the SB system (Jackel et al., 2016).

Functionality of the Wnt and HH reporter plasmids generated were validated by transfecting them with a second doxycycline inducible expression construct

containing coding sequences for either the TCF7, or GLI1 transcription factors, respectively, in the target cells. Derepression of the TetRepressor by 1µg/ml doxycycline yielded the highest *Gaussia*-Luciferase-to-unstimulated-control-ratio after 72h. Functionality was verified in Hek293 (Jackel et al., 2016), iMSC and MSC reporter cells (Figure 20, 23 and 24).

**Canonical Wnt pathway activation**

There are various means available to activate canonical Wnt signaling in vitro, for example, the use of recombinant Wnt proteins, supernatants from Wnt producing cell lines, or the use of lithium chloride (LiCl). More recently, additional small molecule modulators of canonical Wnt signaling have been described (Kahn, 2014). An excellent overview of these agents can be found online at https://web.stanford.edu/group/nusselab/cgi-bin/wnt/smallmolecules.

It was first described in the 1990s that treatment with lithium could activate canonical Wnt signaling through inhibition of the GSK-3β protein kinase (Hedgepeth et al., 1997; Stambolic, Ruel, & Woodgett, 1996). Lithium also exerts effects on other pathways, such as inhibition of inositol monophosphate (IMPase) which causes a reduction in inositol levels and thereby prevents generation of inositol tris-phosphate ($IP_3$) (Berridge, Downes, & Hanley, 1989). Thus, data generated with LiCl treatment may not result exclusively from canonical Wnt signaling. However, it is inexpensive relative to recombinant protein, and was therefore used as an initial screen for the functionality of Wnt reporter cells. Pathway activation was observed in Wnt reporter cells stimulated with medium concentrations (20-25mM) of LiCl as compared to the addition of recombinant Wnt proteins. Depending on the cell type, LiCl toxic effects were observed at varying concentrations. FACS analysis showed increased dead cell numbers as identified with 7-AAD at 4mM LiCl for primary MSCs, whereas iMSCs and Hek293s tolerated higher concentrations. LiCl stimulation in 10% serum-containing medium showed less dead iMSCs compared to stimulation without serum, suggesting that LiCl is less toxic in medium with serum (Data not shown).

As compared to what has been described by others, the experiments detailed here did not show enhanced Wnt reporter activity upon stimulation with 4mM LiCl (Kolben

et al., 2012). This might be due to less sensitive reporter cells, maybe because of higher background of the plasmid's promoter or lower transfection efficiency.

To expand our Wnt-based reagents, Wnt producing expression plasmids were cloned using the Gateway cloning technology and our vector platform developed in part for the present study (Jackel et al., 2016). Coding sequences for the canonical Wnt activators Wnt1, Wnt3 and Wnt3a, were put under control of the constitutively active CMV promoter. Cotransfection of these plasmids into iMSC Bar reporter cells yielded reporter activity induction validating that increased expression of WNT1, WNT3 and WNT3a could induce canonical Wnt signaling in the iMSC (Figure 22). These plasmids are currently being used to further evaluate functionality of Bar reporter cell lines.

**Hedgehog pathway activation**

The HH reporter cells used in this thesis were comprised of a multimer of Gli-responsive elements linked to a minimal promoter driving a reporter gene. Sonic hedgehog (Shh) is the best studied ligand for the HH pathway, however, we refrained from stimulating reporter cells with commercial recombinant Shh as very high, nonphysiologic concentrations were necessary in our preexperiments (as suggested by the manufacturer), and the cost of broad studies was cost prohibited. Shh's physiologic function is dependent on posttranslational modifications and, therefore, the source of the recombinant protein plays an important role. Functionality of the reporter plasmid was verified by cotransfection with an inducible GLI expression construct, but unfortunately no positive control for the whole signaling cascade was readily available. However, substances that could activate our HH reporter cells were identified. These included tumor and macrophage cell line coculture and conditioned supernatant, and the use of human epidermal growth factor (EGF). The HH pathway can be triggered by many intracellular signals, including those mediated by TGF-β, KRAS–MAPK/ERK, PI3K–AKT, IGF, TNF-α induced mTOR/S6K1 activation, and inactivation of hSNF5 (Dennler et al., 2007; Jagani et al., 2010; Z. Ji, Mei, Xie, & Cheng, 2007; Riobo, Lu, Ai, Haines, & Emerson, 2006; Wang et al., 2012). It has been proposed that EGF actions' are mediated by sequential activation of the Shh and Akt signaling transduction pathway (Stepan et al., 2005).

By contrast, iMSC HH could not be activated neither by EGF nor by tumor coculture or supernatant, but could be activated by the induced expression of the Gli transcription factor – positive controls. This suggests that these substances may trigger different signaling cascades in the Hek and iMSC lines which differ in origin and function. Hek cells are human embryonic kidney cells transformed by adenovirus. The iMSCs used in this thesis were L87/4 and V54/2, are SV40 large T-antigen immortalized MSCs derived from bone marrow or the peripheral blood respectively.
It is possible that EGF is a mediator of the effects exerted by macrophage cell line coculture and supernatant. However, more studies, for example with pathway specific inhibitors added to supernatant experiments, are necessary to better understand the mechanisms.

**Wnt and HH responsive reporter MSCs in experimental tumor milieu**

The tumor microenvironment is complex. It is comprised of various normal cells in addition to the tumor cells. These normal cells include; fibroblasts, cells associated with vessels, macrophages and immune cells. The milieu also consists of different zones with varying concentrations of chemokines and growth factors, hypoxia and pH. Due to this complexity, it can be difficult to model it in vitro. In this study, 2D coculture studies, or supernatants harvested from tumor or macrophage cell lines, and 3D invasion assays were applied to characterize the potential activation of Wnt and Hedgehog reporters in engineered MSCs.

To this end, the tumor cell lines SW480 (colorectal adenocarinoma), HT29 (colorectal adenocarinoma), HUH7 (hepatocellular carcinoma), and HT1080 (fibrosarcoma) were chosen after a review of the literature for their general association, and potential to activate either HH or Wnt signaling in the engineered MSC. The presence of the secreted HH ligand, Shh, has been shown by RT-PCR and by Western analysis for SW480 and HT29 (Mazumdar et al., 2011). For HT29, cell surface expression of HH ligands was further validated using FACS analysis (Yauch et al., 2008). Wnt signaling was described using a Wnt-reporter plasmid in SW480 (Pasca di Magliano et al., 2007) and Wnt ligand expression was detected in

HT1080 (Capovilla, 2013). HUH7 as a representative hepatocellular carcinoma cell line, was also included for subsequent experiments.

To investigate the potential influence of the tumor milieu on MSC-reporter induction, 2D coculture was established. The various cell lines tested had different growth characteristics. Viability of the cells was checked through light microscopy, and by use of Höchst-PI to differentiate living from dead cells in fluorescent microscopy. A series of reporter cell-to-tumor-cell-ratios, as well as varying total cell numbers were evaluated. However, the signals derived from the tumor cell lines, or from macrophage cell line coculture were not able to induce the Wnt or HH reporter systems in the engineered iMSCs. By contrast, control Hek293 stably transfected with the aforementioned reporters were strongly induced by the coculture systems suggesting that the MSC may not be as responsive to signaling through the relevant receptors (Figure 25-29).

This was especially true for stimulation of Hek HH reporter cells with HT1080 (human fibrosarcoma cell line) or MM6 (human macrophage cell line)-derived supernatant which led to a roubust induction and to Wnt induction at a much lower level. This suggests that soluble factors secreted by HT1080 and MM6 can exert their effects through HH signaling which in turn may lead to Wnt signaling potentially through pathway cross talk. Alternatively, the supernatant may also contain a small amount of Wnt ligands.

However, the HH or Wnt reporter iMSCs did not show induction upon supernatant exposure, however, for the Wnt-responsive cells a positive control using human recombinant Wnt3a did lead to increased *Gaussia* Luciferase signal. There are various possible explanations for this observation, including differences between Hek293 and iMSC in cell characteristics such as their receptor profile and density, or even transfection efficiency. The response of the Hek Wnt reporter cells to Wnt3a stimulation yielded higher fold changes to unstimulated control than was seen in the iMSCs exposed to the same concentration of Wnt3a.

Concentrated MM6 supernatant (Amicon centrifugal filter 10kDalton, concentrating proteins) increased the Hek reporter signal significantly as compared to unconcentrated supernatant, or to unstimulated controls. By contrast, the iMSC reporter cells showed rather decreased reporter activity. This suggests differences between Hek and iMSC lines in their response to the MM6 supernatant. There are

various potential reasons for this effect, including differences in the signaling pathways. One possibility is that these pathways may be more active in the “resting” iMSC, and further stimulation leads to downregulation of that pathway. The receptor profile may also differ such that a given signal triggers different signaling cascades in the cell lines leading either to activation or repression of reporter gene expression.
To better define the activation of the Hek reporter cells with MM6 supernatant, various substances were tested for their potential to activate Hek HH cell reporter activity, or to reverse stimulatory effects of the MM6 coculture or supernatant. Human epidermal growth factor (EGF) was found to be able to activate the Hek HH reporter, but did not activate iMSC HH. Crosstalk between the HH cascade and EGFR signaling has been previously reported (Bigelow, Jen, Delehedde, Chari, & McDonnell, 2005; Kasper et al., 2006; Schnidar et al., 2009).

A 3D tumor model, or so-called tumor spheroid, was also tested in this setting. It was thought that it may generate a set of signals that are more in tune with the in vivo tumor environment. The analysis of tumor spheroids and their interaction with MSCs was found to be time-consuming and complex. Therefore, spheroid invasion assays were not suitable for the general screening of prestimulation conditions. Notwithstanding, testing pathway specific reporters for their activity in different regions of the tumor spheroid with its different gradients of chemokines and hypoxia would be interesting and therefore, establishing a suitable iMSC cell line was aspired.

**Establishment of a Wnt-responsive cell line with fluorescent protein read-out**

Therapeutic approaches utilizing engineered MSCs are dependent on the expression of therapeutic transgenes within the tumor milieu. In order to minimize potential side effects when the MSC are recruited to normal tissue, the transgene expression can be made more specific for the tumor environment thus limiting potential damage to the normal tissues.

To investigate the tumor milieu and its potential effect on MSCs reporter cells, a fluorescent protein read-out was established to measure promoter activity of stably transfected MSCs while invading a 3D tumor model. A co-worker in our laboratory,

Svenja Rühland describes in her Master's thesis, MSC invasion into tumor spheroids using a mathematical approach to analyze the depth of invasion (Rühland, 2014). Stably transfected iMSC with the Bar-Tomato reporter were subsequently shown to invade a HUH7 spheroid, Tomato signal and CMFDA label were detectable (Figure 32).

The functionality of stably transfected iMSCs with a Wnt responsive element driving Tomato expression was verified in fluorescent microscopy and FACS analysis. Stimulation for 24h with recombinant human Wnt3a (75 and 400ng/ml) and LiCl (25mM) lead to more Tomato positive cells as well as a higher y-mean in FACS analysis (Figure 33). Unfortunately, only a small percentage of cells reacted to the more physiologic canonical Wnt pathway stimulator Wnt3a. In accordance with the *Gaussia* Luciferase Wnt reporter experiments, neither HUH7 nor HT1080 tumor cell supernatant was able to induce reporter activity in iMSC nor primary MSC Wnt-Tomato reporter cells (Figure 34).

In conclusion, stimulation of Wnt responsive elements stably transfected in iMSC or primary MSC did not lead to induction within experimental tumor milieus. Therefore, the generated cell line was not suitable for establishing this assay, and the results suggest that MSC do not respond strongly to HH or Wnt signals within tumors. The current standard using the CCL5/RANTES promoter to drive therapeutic transgene expression in engineered MSCs (currently in Phase I/II trials) makes use of inflammatory signals within the tumor setting to trigger transgene expression. The results presented here suggest that Wnt or HH signaling may not be suitable for driving tumor-specific transgene expression in engineered MSC. However, the preactivation studies, do suggest that stimulation of MSC with canonical Wnt ligands (here Wnt3a) may transiently enhance their tropism for tumor-derived signals.

## Conclusion & outlook

- Wnt3a prestimulation enhanced primary MSCs' migratory potential towards tumor conditioned medium.
- No memory effect was observed, MSCs cultured for two to three passages after preconditioning did not show enhanced migratory parameters.
- It was possible to activate Wnt and Hedgehog pathway specific promoters in the tumor milieu, stably transfected into Hek293 cells.
- MSCs and iMSCs stably transfected with Wnt or Hedgehog pathway specific promoters did not show enhanced activity in the tumor milieu.
- When exposed to concentrated supernatant of a macrophage cell line, iMSC Wnt or Hedgehog reporter cells showed decreased reporter activity, in contrast to the control Hek reporter cells which showed good induction.

The Wnt- and HH-responsive elements used in this thesis do not appear to be suitable for the application as enhancer/promoter system for selectively driving therapeutic gene expression in MSCs, as no induction in the experimental tumor milieu was observed. Further screening of potential enhancers/promoters is necessary to identify the most suitable gene promoters for the therapeutic application.

Further experiments are needed to evaluate the potential of Wnt3a prestimulation of MSCs not only in vitro, but also in vivo. Being able to influence MSC directed migration towards tumor-derived signals helps us to understand MSC biology better as well as opens doors for improving their clinical application.

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## Abbreviations and Symbols

| | |
|---|---|
| 7-AAD | 7-Aminoactinomycin D |
| ANGPT2 | Angiopoietin 2 |
| att | Gateway cloning sites |
| Bar | β-catenin activated reporter, Wnt-responsive element in canonical Wnt repoter plasmids |
| bp | Base pairs |
| BSA | Bovine serum albumin |
| CAF | Carcinoma associated fibroblasts |
| CCL2 | Chemokine (C-C motif) ligand 2 |
| CCL5/RANTES | Chemokine (C-C motif) ligand 5/regulated on activation, normal T cell expressed and secreted |
| CD | Cluster of differentiation |
| CDS | Coding sequence |
| CEA | Carcinoembryonic antigen |
| CK1-α | Casein kinase 1α |
| CM | Conditioned-medium |
| CMFDA | 5-Chloromethylfluorescein diacetate |
| CXCR4 | C-X-C chemokine receptor type 4 |
| Dhh | Desert hedgehog, Hedgehog signaling |
| DMEM | Dulbecco's Modified Eagle's Medium |
| DMSO | Dimethylsulfoxide |
| Dox | Doxycycline |
| *E.coli* | *Escherichia coli* |
| EMT | Epithelial-mesenchymal transition |
| FACS | Fluorescence-activated cell sorting |
| FCS | Fetal calf serum |
| FMI | Forward migration index, a calculated parameter indicating directed cell migration |
| Fz | Frizzled, G protein-coupled receptor proteins |
| GCV | Ganciclovir |
| GDEPT | Gene-directed enzyme-producing therapy |
| Gli | Glioma-associated oncogen, Hedgehog signaling |
| GSK-3β | Glycogen synthase kinase |
| hbMSC | Human bone marrow derived mesenchymal stem cell; primary cells |
| hbMSCApp172_1 | name of primary mesenchymal stem cells used for experiments |

| | |
|---|---|
| Hek293 | Human embryonic kidney cell; well characterized cell line to conduct proof of principle experiments |
| HGF | Hepatocyte growth factor |
| HH | Hedgehog |
| HSV-tk | Herpes simplex thymidine kinase |
| HT1080 | Human fibrosarcoma cell line |
| HT29 | Human colorectal adenocarcinoma cell line |
| HUH7 | Hepatocellular carcinoma cell line |
| Ihh | Indian hedgehog, Hedgehog signaling |
| IL-8 | Interleukin-8 |
| iMSC | Immortalized mesenchymal stem cell line |
| ITR | Inverted terminal repeats |
| LiCl | Lithium chloride |
| MDC | Macrophage-derived chemokine |
| MIF | Migration inhibitory factor |
| MM6 | Monocytic tumor cell line |
| MMP-2 | Matrix metallo- proteinase-2 |
| MSC | Mesenchymal stem cell |
| NIS | Sodium iodide symporter |
| Ori | Origen of replication |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase chain reaction |
| PEDF | Human pigment epithelium-derived factor |
| PI | Propidium iodide |
| PKA | Protein kinase A |
| Ptch1 | Protein patched homolog 1, in mice and humans, Hedgehog signaling |
| Rantes | Regulated on activation, normal T cell expressed and secreted |
| RLU | Relative luciferase unit |
| SB100 | Sleeping Beauty 100 Transposase |
| SDF-1 | Stromal-derived factor |
| Shh | Sonic hedgehog, Hedgehog signaling |
| Smo | Smoothened, Hedgehog signaling |
| SUFU | Suppressor of fused |
| SW480 | Human colorectal adenocarcinoma cell line |
| Tet | Tetracycline |
| TNFα | Tumor necrosis factor α |
| VEGF | Vascular endothelial growth factor |

# Eidesstattliche Versicherung

Kraus, Christiane

Name, Vorname

Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

**Engineered Mesenchymal Stem Cells as therapeutic Vehicles for the Treatment of solid Tumors: Wnt and Hedgehog biology-based Targeting and transgene Expression**

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